STRATEGIES TO CULTIVATE MICROALGAE ON EUTROPHIC WASTEWATER FOR NUTRIENTS RECYCLING AND BIOMASS PRODUCTION

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Abstract

Wastewater generated from industry may contain excessive nutrients, including nitrogen, phosphorus and organic carbon. On one hand, excessive nutrients in wastewater could cause environmental pollution and ecological disaster. On the other hand, these nutrients could be utilized for algae growth and algal biomass production. Unregulated discharge of eutrophic wastewater not only poses threats to water body, but also wastes the valuable nutrients in wastewater. This dissertation research focuses on the technologies and mechanisms to improve the efficiency of nutrients utilization by algae grown in eutrophic wastewater.

The lack of ammonia limits algae growth in wastewater from food industry. In this study, a potential solution is to mix the wastewater from different resources to balance the nutrients profiles and promote the algae growth. The results showed that appropriate mixture of food industry wastewater effectively mitigated the bottleneck to algae growth and improved the nutrients removal efficiencies.

Ammonia toxicity is a serious concern in the treatment of some wastewater. In this study, comparison of three common carbon sources, glucose, citric acid, and sodium bicarbonate, indicated that in terms of ammonia assimilation, glucose is the best carbon source. This result could be applied to the toxicity of ammonia enrichment to algae cultivation in eutrophic wastewater.

A cooperation model between algae and wastewater-borne bacteria was reported by this dissertation research. Such a cooperation model increased the nutrients removal efficiencies and promoted the algae growth. A strain of beneficial aerobic bacteria, *Acinetobacter* sp., was isolated and its biochemical characteristics were explored. After

treatment by co-cultivation of *Acinetobacter* sp. and *Chlorella* sp., residual nutrients in municipal wastewater were reduced to be under the permissible discharge limit.

To fully utilize the nutrients in swine manure, it is always exploited to produce biogas by anaerobic digestion. However, the treatment of residual nutrients after anaerobic digestion is a critical issue. High turbidity and ammonia toxicity are two factors limiting the algae growth in anaerobically digested swine manure. This research developed a strategy to pretreat the anaerobically digested swine manure by cationic starch-assisted turbidity reduction and air bubbling-driven ammonia stripping.

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Chapter 1. Introduction

1.1. Background

Food and energy shortages have caused serious social problems worldwide (Brown, 2009). Furthermore, the population explosion is leading to the dramatic increase in food and energy demand (Rosegrant & Cline, 2003). Traditional crops, such as wheat and soybean, could not solve the food or energy shortage problems because of their long cultivation period and low biomass accumulation rate (Clarens et al., 2010; Umdu et al., 2009). In addition, cultivation of traditional crops is impacted by climate changes and seasons (Altieri & Koohafkan, 2008). To solve these problems, researchers are trying to find new biomass resources and develop biomass products at low cost.

Food industry provides various product choices to consumers but also produces large amount of wastes (Oh & Logan, 2005). Previous studies and preliminary data of this study indicated that wastewater from food processing industry contained various nutrients, such as organic carbon, phosphorous, and protein (De-Bashan & Bashan, 2004; Obaja et al., 2005). In addition, concentrate municipal wastewater is also considered as a type of eutrophic wastewater. These nutrients in wastewater could promote the growth of toxic microorganism in water body, causing water pollution (Tam & Wong, 1989). If wastewater could be used as a raw substrate for biomass production, cost for both waste treatment and biomass production would be reduced significantly and the potential threats of wastewater to the environment could be mitigated.

1.2. Algae as a new biomass resource

Algae, which are usually unicellular plant-like microorganisms, contain protein, lipid, saccharide, vitamins, and dietary fiber (De Roeck-Holtzhauer et al., 1991; Zhou et al., 2012c). Research conducted so far showed that algal biomass has promising qualities as a new source of biomass. For example, some algal species could synthesize highly valuable ingredients, such as unsaturated fatty acids (Arterburn et al., 2006). The concentration of different nutrients in algae depends on the algal species, growth environment, and many other factors. Algae grown on media with high concentration of organic carbon are prone to lipid synthesis while algae in phototrophic growth model have lower level of lipid (Liu et al., 2011). Furthermore, algae grown in the environment with sufficient nitrogen would synthesize more protein while under nitrogen deficiency condition algae tend to synthesize more lipids as storage energy (Bar et al., 1995; Wang et al., 2009). To produce biomass through algae cultivation, various factors, including algal strains, growth environment and so forth, can be manipulated and optimized.

In terms of biomass production, algae have three important advantages: (1) algae have much higher (5-30 times) biomass yield than traditional crops per unit surface area; (2) algae production which can be conducted on waste or non-arable land does not use traditional agricultural resources, particularly farm land; and (3) some algal strains contain high contents of protein or lipid which is important resource in biomass industry. Therefore, algae are regarded as an important resource for biomass production.

1.3. Wastewater

Fast development of industry produces much wastewater which poses serious threats to environment (Oh & Logan, 2005). Municipal wastewater and food processing wastewater are two common types of wastewater. Previous studies showed that wastewater from different industries have very different characteristics. For example, wastewater from sugar processing industry contains high concentration of COD due to the organic carbon left in the wastewater (Hamoda & Al-Sharekh, 1999). Wastewater from meat processing industry contains high concentration of nitrogen since meat residual in wastewater releases nitrogen (Van Oostrom, 1995). Cultivation of algae using wastewater is considered a sustainable pathway to prevent the environmental pollution caused by wastewater since algae could remove some of the nutrients in wastewater. In addition, nutrients in wastewater could be converted into valuable biomass by algae.

Previous studies have tried to cultivate algae in concentrate municipal wastewater and food processing wastewater. However, some problems, such as ammonia toxicity, low nutrients removal efficiencies, and negative effects of wastewater-borne bacteria on algae growth, have not been solved yet. To promote the application of algae technology in the treatment of municipal wastewater and food processing wastewater, in this research, problems associated with the cultivation of algae using food processing wastewater and municipal wastewater will be investigated and possible solutions will be proposed.

1.4. Objectives

The overall goal of this study was to investigate cultivation of microalgae using different kinds of wastewater for simultaneous nutrient removal and biomass production. The specific objectives were:

(1) To balance nutrients profiles of food industry wastewater for algae cultivation and nutrients removal

(2) To assess the effects of different carbon sources on ammonia removal in wastewater and explore potential metabolic mechanisms

(3) To apply algae technology to wastewater treatment at pilot scale and identify the beneficial bacteria in algal-bacterial system

(4) To pretreat waste effluent by cationic starch-assisted turbidity reduction and air bubbling-driven ammonia stripping

Chapter 2. Literature review

2.1. Algae cultivation in wastewater

Microalgae have the potential to become an important protein and oil source for animal feeds, human diets, and fuels because of their high productivity (Vigani et al., 2015). Commercial large scale production of algae is expected to help address the worldwide food and energy shortage concerns. However, current algae technologies are mostly unsustainable and expensive. Most commercial algae cultivation systems use synthetic chemicals as nutrient source for algae growth. High price of synthetic chemicals is one of the critical factors which increased the production cost of algal biomass and limited its wide application in practice (Slade & Bauen, 2013). Replacing the expensive synthetic chemicals with cheap resources as nutrient source is a promising way to reduce the cost of algae technologies.

The early interest in algae cultivation can be traced back to the use of algae to treat wastewater. The benefits of using algae to treat different types of wastewater have been documented in numerous research reports (Christenson & Sims, 2011; El-Sikaily et al., 2007; Li et al., 2011b). Nutrients, including nitrogen, phosphorus, and organic carbon, in wastewater are partly responsible for the environmental pollution and ecological disaster (Farooq et al., 2013; Grothe & Park, 2000). The cyanobacteria blooms, which would consume oxygen in water body and cause ecological disasters, are mainly caused by the discharge of eutrophic wastewater into water body (Li et al., 2011a). It was reported that in England and United States, the annual economic losses caused by eutrophication of freshwater reached $160 million and $2.2 billion, respectively (Dodds et al., 2008; Pretty et al., 2003). Cultivation of algae on eutrophic wastewater is considered a pathway to

sustainable production of algal biomass and wastewater treatment because it reduces the production cost and generates environmental benefits (Norsker et al., 2011). Previous studies showed that algae could grow on different types of eutrophic wastewater, including municipal wastewater, animal manure, and industrial wastewater, which are available at no or very low cost (Su et al., 2012). However, because of some technical bottlenecks, until now, algae technology has not been widely applied in large-scale treatment of wastewater.

As shown in Table 2.1, biomass yields of algae in some wastewater are low. In some cases, due to the limitation of algae growth, nutrients removal efficiencies in wastewater were kept in a low level. To further promote the application of algae technologies in wastewater treatment, specific technical bottlenecks should be identified and solved. Firstly, deficiency of certain nutrient in wastewater may prohibit the algae growth and further limit the removal of other nutrients. Secondly, some wastewater-borne bacteria pose threats to algae growth and even cause the failure of algae cultivation. The competition of bacteria with algae for nutrients was reported by previous study (Ramanan et al., 2016). Thirdly, ammonia toxicity is another technical bottleneck to algae cultivation in some eutrophic wastewater. It was reported that high concentration of ammonia in wastewater negatively impacted the photosynthesis efficiency (Collos & Harrison, 2014).

Table 2.1. Microalgae growth in wastewater

Wastewater	Nutrient concentration (mg/L)			Yield of algal biomass (g/L)	Period (days)	Reference
	TN	TP	COD			
Carpet industry wastewater	NA	3.47-7.89	106-183	0.34	9	(Chinnasamy et al., 2010)
Chicken manure	NA	NA	NA	0.60	NA	(Cheung & Wong, 1981)
Concentrated municipal wastewater	134	212	2324	0.9	9	(Zhou et al., 2011)
Dairy wastewater	36.6	1.8	NA	0.5	9	(Woertz et al., 2009a)
Industrial and municipal wastewater	NA	NA	NA	0.21	NA; 6% CO_2 supplied	(Chinnasamy et al., 2009)
Municipal wastewater	51	2.1	NA	0.84	4; (CO_2 supplied)	(Woertz et al., 2009a)

2.2. Metabolisms of nutrients in algal cells

One of the main differences between artificial culture medium and wastewater is that wastewater is commonly imbalanced in nutrient. For example, dairy wastewater contains

excessive concentrations of organic carbon while have low contents of nitrogen (Zinadini et al., 2015). As discussed above, deficiency of certain nutrient in wastewater is one of the technical problems associated with algae cultivation. In this section, studies on metabolisms of two major nutrients, including nitrogen and organic carbon, in algal cells were reviewed.

2.2.1. Metabolism of nitrogen

Since algal cells without nitrogenase could not assimilate nitrogen gas in air, the major nitrogen source for algae growth is from culture medium. It was reported that in wastewater the major nitrogen sources include nitrate, nitrite, ammonia, and some organic nitrogen, such as protein degradation products, urea, and amino acids (Kim et al., 2013). Production procedure in industries is the main factor that determines the nitrogen profile of wastewater. For example, in this work, some meat processing wastewater contains high content of nitrogen due to the existence of protein degradation products. Nitrogen, which is an essential element for the synthesis of amino acids, energy transfer molecule (ATP), and DNA, plays a critical role in algal metabolisms (Dodds et al., 2002). Therefore, to improve the protein content in algal biomass or promote the algal metabolisms, nitrogen absorption and assimilation in algal cells should be maximized.

Assisted by the specific transport proteins on algal cells, some nitrogen sources in culture medium or wastewater are transported into algal cells through high affinity transport system or low affinity transport system (Zhou et al., 2000). As shown in Figure 2.1, assisted by enzymes, such as urease, nitrite reductase, and nitrate reductase, different nitrogen sources absorbed by algal cells are converted into ammonia, which is used for

further synthesis of glutamate. The main pathway for ammonia assimilation in algal cells is glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT), which is assisted by both glutamine synthetase and glutamine oxoglutarate aminotransferase (Inokuchi et al., 2002). In the GS-GOGAT pathway, biochemical reactions are driven by ATP and NADH, which are from glycolysis, Krebs cycle, or other reactions. Therefore, nitrogen assimilation is closely associated with some other metabolisms, particularly carbon metabolisms. Final product of GS-GOGAT pathway is glutamate, which is a substrate for protein synthesis.

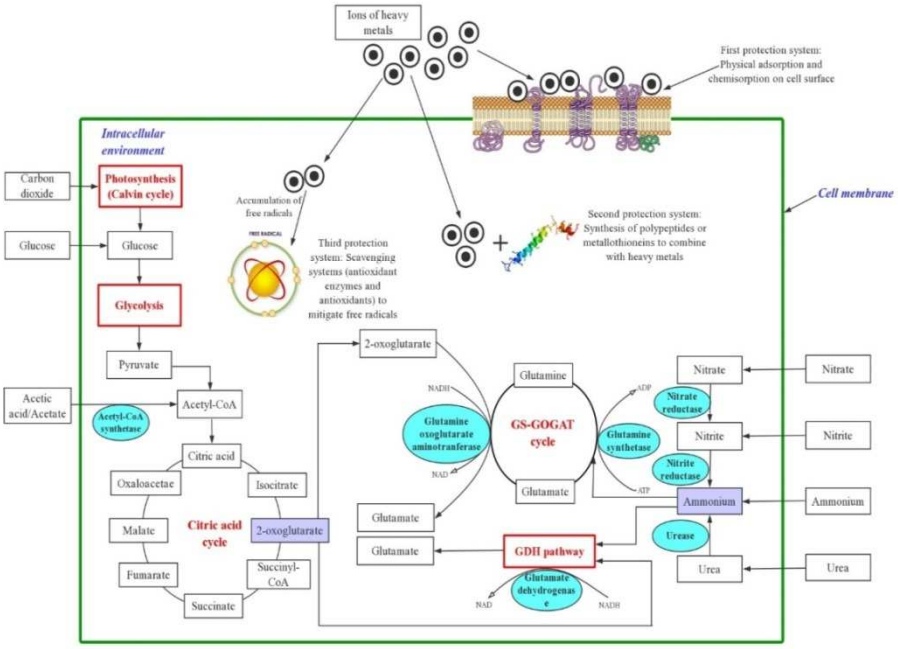


Figure 2.1. Assimilation of nutrients and heavy metals in wastewater through algal metabolism

Considering the importance of nitrogen in algal metabolisms, various factors that have significant effects on nitrogen absorption and assimilation have been explored. Firstly, the transport proteins on cell membrane determine the absorption process of nitrogen (Liu

et al., 2003). Secondly, activities of enzymes, including urease, nitrite reductase, and nitrate reductase, which impact the conversion of nitrogen sources into ammonia, should be improved (Glass et al., 2009). Thirdly, the biochemical reactions of GS-GOGAT pathway should be promoted to realize the conversion of ammonia to glutamate. In order to promote the nitrogen absorption and assimilation, both endogenous method and exogenous method have been developed. For instance, nitrate reductase gene was introduced into green alga, *Dunaliella viridis*, to promote the assimilation of nitrate (Sun et al., 2006). However, due to the use of genetic technologies, in practice, endogenous method is not always used for algae biomass production. The exogenous methods include nitrogen starvation pretreatment, balancing the nutrients profile in medium, and optimization of cultivation temperature.

According to previous studies, balancing the C/N ratio in medium is an effective way to promote the nitrogen assimilation. Previous study showed that in culture medium, the ratio of C/N should be controlled at 6:1 (Sanudo-Wilhelmy et al., 2004). Although this theory is not applicable in some wastewater, it still demonstrates the importance of C/N ratio in algal metabolisms. In algal cells, Krebs cycle produces 2-oxoglutarate, which is an essential substrate for ammonia assimilation. Low carbon content in wastewater would inhibit the generation of 2-oxoglutarate. Therefore, to maximize the nitrogen assimilation, C/N ratio in wastewater should be balanced.

2.2.2. Metabolisms of organic carbon

Some solid carbon in wastewater could not be absorbed by algae due to the large sizes. It was reported that in municipal wastewater and oil crop wastewater, dry weights of solid

particles reached 1.12 g/L and 1.26 g/L (Liu et al., 2017). Discard of these solid particles would not only waste nutrients in wastewater, but also cause potential pollution. Therefore, releasing carbon source in solid particles was a hot research topic in previous studies. Anaerobic digestion has been successfully applied in the conversion of solid particles into soluble nutrients (Bolzonella et al., 2005).

Soluble carbon sources in wastewater could be classified into two types, organic carbon and inorganic carbon. Common inorganic carbon sources include carbonate, bicarbonate, and carbon dioxide, while organic carbon sources are complicated. Organic carbon sources which could be efficiently utilized by algae include volatile fatty acids and some soluble saccharides. Algal photosynthesis, which is mainly responsible for the fixation of carbon dioxide, has been fully documented by previous studies. The final product of photosynthesis, glucose, is the precursor for glycolysis, a series of reactions to generate ATP and NADH. Due to the different metabolic pathways and enzyme activities, algal cells may prefer certain carbon source over other carbon sources (Ren et al., 2013). To promote nutrients removal in the wastewater treatment, appropriate algal strains should be used based on the algal metabolic pathways and nutrients profile of wastewater.

2.3. Interaction between algae and wastewater-borne bacteria

In wastewater treatment plant, it is not economically feasible to sterilize the wastewater before inoculation of algae. In previous studies, the existence of bacteria in wastewater has been proven to pose threats to algae growth or even cause the failure of algae cultivation (Zhang et al., 2012). Firstly, bacteria could compete with algae for nutrients in wastewater. As a result, deficiency of certain nutrients in wastewater would limit the

algae growth. Secondly, some bacterial species could cause the failure of algae cultivation by releasing toxic components. However, recently, three cooperation models between algae and bacteria have been reported by some studies (Su et al., 2012).

Firstly, the co-cultivation model is established on the transfer of carbon dioxide and oxygen between bacteria and algae (Munoz & Guieysse, 2006). In such a cooperation model, oxygen produced by algae through photosynthesis is favorable to bacterial metabolisms and carbon dioxide produced by bacteria could be utilized by algae as carbon source. An artificial algal-bacterial community by co-immobilizing *Azospirillum brasilense*, which could release carbon dioxide in wastewater, and microalgae, has been developed (De-Bashan et al., 2004). Secondly, extracellular enzymes, such as lipase and protease, released by bacteria could convert some large solid particles in wastewater into digestible nutrients (Falony et al., 2006). Accordingly, algae are supposed to have higher biomass yield in wastewater with more digestible nutrients. Thirdly, some special bacteria could release vitamins, which are growth-promoting factors of algae (Croft et al., 2005).

In wastewater treatment, the establishment of cooperation between algae and wastewater-borne bacteria would make the sterilization step unnecessary. Accordingly, the treatment procedure could be simplified and costs of wastewater treatment could be reduced. Previous studies have discovered that the cooperation between algae and wastewater-borne bacteria in municipal wastewater increased algae growth rate and nutrients removal efficiency (Lee et al., 2015). However, specific bacterial strains having favorable effects on algae growth have not been fully explored in wastewater-borne bacterial community.

2.4. Ammonia toxicity in wastewater

Ammonia, which participates in protein synthesis, is an essential nutrient for algae growth and intracellular metabolisms. As shown in Figure 2.1, ammonia is fixed through glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway for further amino acids synthesis. The lack of ammonia in wastewater would reduce the protein content in algal cells and limit the intracellular metabolisms. However, excessive ammonia in culture medium would cause ammonia toxicity (Nimptsch & Pflugmacher, 2007). According to previous studies, inhibitory NH_3-N concentrations for Chlorophyceae, Diatomophyceae, Dinophyceae, Prymnesiophyceae, and Raphidophyceae are 23758, 725, 324, 958, and 635 μM, respectively (Collos & Harrison, 2014).

A couple of mechanisms have been proposed to explain the ammonia toxicity to algal cells. Firstly, high content of ammonia could reduce the photosynthesis efficiency by damaging the PSII system in algal cells (Dai et al., 2012). Secondly, intracellular oxidative stress triggered by excessive ammonia would inhibit activities of some enzymes and even cause lipid peroxidation (Nimptsch & Pflugmacher, 2007). When the intracellular oxidative stress exceeded tolerance, cell-level disorder will occur and algae growth will be prohibited (Choo et al., 2004). As ammonia toxicity has very complex mechanisms, further studies will add additional point of view to explain the algal metabolisms associated with ammonia toxicity (Nimptsch & Pflugmacher, 2007).

Alleviating ammonia toxicity in wastewater is important to the application of algae technology in wastewater treatment. Many studies reduced concentrations of NH_3-N in wastewater before algae inoculation (Park & Kim, 2015; Serna-Maza et al., 2014).

Common pretreatment methods for NH_3-N removal in wastewater include dilution by freshwater and ammonia stripping. After pretreatment, concentration of NH_3-N in wastewater could be reduced to a lower level and the ammonia toxicity is mitigated (Lu et al., 2016). However, some disadvantages have been observed in these methods. For example, since wastewater dilution needs high volume of freshwater, it is not regarded as a sustainable way for the wastewater treatment at large scale. In the process of ammonia striping, removal of ammonia from wastewater wastes the nutrients and reduces the nutrients utilization efficiencies. In addition, gaseous ammonia discharged into atmosphere may not be compliant with air quality regulations (Guštin & Marinšek-Logar, 2011). Because of these disadvantages, ammonia toxicity to algae growth is still a technical problem for the sustainable treatment of wastewater with high concentrations of NH_3-N and has to be alleviated.

Chapter 3. Use of dairy wastewater in algae culture by nutrients balancing

3.1. Introduction

In the United States, the annual milk yield was improved from 53.1 billion kg in 1944 to 84.2 billion kg in 2007 (Capper et al., 2009). The fast development of dairy processing industry is producing increasing amounts of dairy wastewater, which has been regarded as a source of water body pollution. Researchers have demonstrated that dairy processing wastewater with high concentrations of organic carbon, nitrogen and phosphorous, which could be utilized by different types of microorganisms, was the chief cause of water pollution in some areas (Yu et al., 2014). The traditional treatment strategies of dairy processing wastewater included aerobic digestion and anaerobic digestion. In recent years, there has been an increasing interest in growing microalgae on dairy processing wastewater to produce biomass and remove nutrients (El-Sikaily et al., 2007). The treatment based on microalgae cultivation had low cost of infrastructure and could produce biomass with many valuable compositions, such as protein, lipid, and antioxidants. In addition, in comparison with other sources of wastewater, such as municipal wastewater and animal manure, dairy processing wastewater had more nutrient, particularly organic carbon, which is important to algae growth.

Researchers have successfully used dairy processing wastewater to grow algae. But one problem is that biomass yield of algae in dairy processing wastewater is very low. It has been proven that the highest biomass yield of microalgae in dairy processing wastewater was lower than 0.7 g/L (Blier et al., 1995; El-Sikaily et al., 2007). Dairy processing wastewater had high contents of biological oxygen demand (BOD) (500-4500 mg/L) and chemical oxygen demand (COD) (950-7500 mg/L) (Christenson & Sims, 2011). The

concentration of COD in dairy whey even reached 69000 mg/L (Öztürk et al., 1993). Woertz et al. (2009) diluted dairy wastewater by 10% before the algae cultivation to prevent the negative effects of COD on microalgae growth. However, the biomass yield was lower than 0.6 g/L (Woertz et al., 2009b). Dairy processing wastewater also had different types of metals (Markou & Georgakakis, 2011). The average concentration of ammonia nitrogen (NH_3-N) in wastewater was lower than 50 mg/L. In some dairy processing wastewater, the NH_3-N contents were even lower than 5 mg/L (Longhurst et al., 2000). Previous study showed that NH_3-N in wastewater could be totally utilized by microalgae in three days (Lincoln et al., 1996). Therefore, the ammonia deficiency should be the factor limiting algae growth in wastewater, including dairy processing wastewater. By adding dairy final effluent into pulp and paper influent, previous study balanced the nutrient profile of wastewater for algae cultivation and produced more biomass (Gentili, 2014). This study suggested that wastewater mixing is a possible method to promote algae growth and increase nutrients removal.

The main aim of this chapter was to develop a cheap and efficient method to improve the biomass yield of algae in dairy processing wastewater. The specific steps in this chapter include: (1) Measuring the nutrient profile and metal element profile in dairy processing wastewater; (2) Exploring nutrient removal and microalgae growth in dairy processing wastewater without mixing pretreatment; (3) Identifying the factors limiting algae growth in dairy processing wastewater; (4) Mixing dairy wastewater with another wastewater with high concentration of ammonia to promote the nutrient removal at low cost.

3.2. Materials and methods

3.2.1. Materials and chemicals

Three kinds of wastewater, including salt whey, mother liquor, and liquid whey, were obtained from different processing steps in a dairy processing plant in Minnesota, USA. The analysis of ammonia nitrogen (NH_3-N), total nitrogen (TN), chemical oxygen demand (COD), and total phosphorous (TP) was conducted by using assay kits obtained from Hach (USA). Before algae inoculation, the wastewater was centrifuged for 10 min and autoclaved at 121°C for 30 min.

3.2.2. Growth and chemical analysis

3.2.2.1. Algae growth and nutrient recovery

Microalgae were cultivated in 250-mL flasks with artificial medium or different types of wastewater. Initial density of microalgae in wastewater or artificial medium was about 0.25 g/L.

The biomass yield was analyzed daily according to previous publication (Zhou et al., 2012b). The average growth rate was calculated accordingly.

$$R = (W_t - W_0)/t \quad \text{Eq. 1}$$

where R is the growth rate of microalgae based on biomass yield; *t* is the time interval (days); W_t and W_0 are the biomass yield on Day t and Day 0, respectively.

A linear model (Eq. 2) was used to describe the relationship between algae growth and biomass density (Yang et al., 2011a).

$$N = K/(1 + e^{a-rt}) \quad \text{Eq. 2}$$

where N (mg/L) is the dry weight of algal biomass on Day t; K (mg/L) is the maximum biomass accumulated in the culture; a is a constant; and r (day^{-1}) is the specific growth rate.

3.2.2.2. Nutrient profile analysis

Nutrient profiles of dairy processing wastewater were analyzed by using a Hach DR 5000 Spectrophotometer based on the previous publication (Li et al., 2011b).

3.2.2.3. Analysis of protein and lipid in microalgae

In this work, nitrogen-to-protein conversion factor (NTP) of 6.25 was used for the calculation of protein content (Dominguez, 2013).

Harvested algae biomass was dried in a vacuum dryer (Hu et al., 2013). Total lipid in microalgae was measured according to the method described by Folch et al. (Folch et al., 1957).

3.3. Results and discussion

3.3.1. Dairy wastewater characteristics

TN, NH_3-N, COD, and TP in wastewater and artificial medium were shown in Table 3.1. The results suggested that the dairy processing wastewater had very high contents of TP and COD. The most possible reason for this phenomenon is that dairy processing wastewater has a lot of organic carbon. A too high concentration of organic carbon may

seriously limit the algae growth (Wang et al., 2010). In this chapter, the dairy processing wastewater was diluted before algae inoculation.

Table 3.1. Nutrient profiles of dairy wastewater

	NH_3-N (mg/L)	TN (mg/L)	TP (mg/L)	COD (mg/L)
Mother liquor	429	3570	22350	191000
Salt whey	57.8	935	735	30700
Liquid whey	24.8	2164	1012.5	76350
TAP medium	132.0	364.4	28.6	3870
Mother liquor (mixed)	151.3	281.3	565.3	6000
Salt whey (mixed)	151.7	322.9	59.47	3130
Liquid whey (mixed)	172.3	351.6	157.0	4693

As shown in Table 3.2, dairy processing wastewater had very high concentrations of some metal elements. It was reported that the microalga growth may be limited by the high concentrations of metals. In this chapter, to prevent the potential negative effects of high concentrations of some metal elements on algae growth, the dairy processing wastewater was diluted before algae cultivation. According to the metal profile analysis, dairy processing wastewater with these essential metal elements should be a medium alternative for algae cultivation (Davis et al., 2003). Dilution of dairy processing wastewater will minimize the negative effects of some metal elements on algae growth.

Table 3.2. Metal profiles of dairy wastewater

mg/L	Mother liquor	Salt whey	Liquid whey	TAP medium
B	1.77	0.22	0.11	2.02
Ca	1726.00	983.90	329.20	13.60
Co	< 0.01	< 0.01	< 0.01	0.40
Cu	< 0.02	< 0.02	< 0.02	0.40
Fe	0.33	0.17	0.25	1.00
K	1407.00	69.46	94.85	63.90
Mg	615.20	99.65	57.01	9.76
Mn	< 0.01	< 0.01	< 0.01	1.41
Mo	0.22	< 0.01	< 0.01	0.60
Na	3400.00	2462.00	112.60	6.18
Zn	1.08	0.34	0.17	4.93

3.3.2. Algae growth and nutrient removal

3.3.2.1. Optimization of dilution rate

In the preliminary experiment, algae did not have any growth in wastewater without dilution. Therefore, the dairy processing wastewater was diluted by different ratios before algae cultivation. The dilution seriously reduced the concentrations of NH_3-N in dairy processing wastewater. The biomass yields of algae in dairy processing wastewater at different dilutions rate were shown in Figure 3.1.

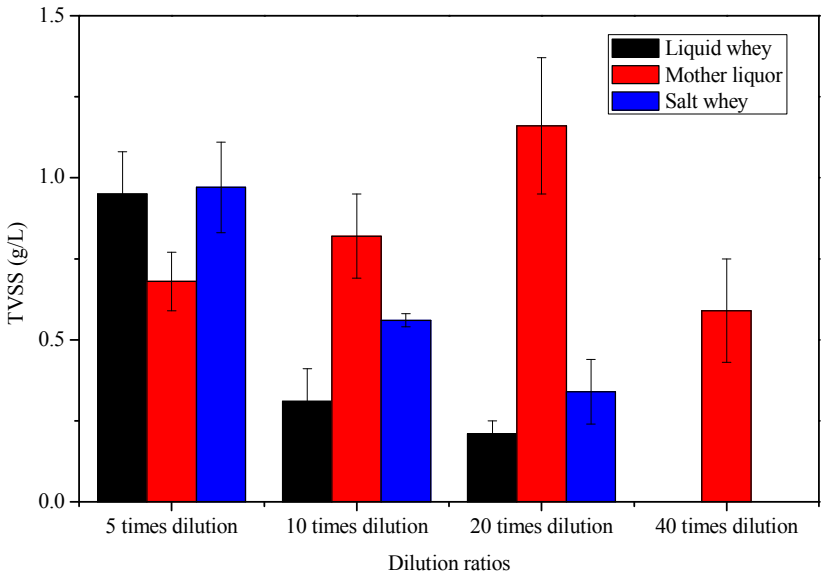


Figure 3.1. Growth of algae in wastewater with different dilution rates

Algae in liquid whey had the highest biomass yield (0.95 g/L) at 5 times dilution In addition, algae in salt whey had the highest biomass yield (0.97 g/L) at 5 times dilution. The optimum dilution rate of mother liquor for algae growth should be 20 times at which the biomass yield reached 1.16 g/L. In mother liquor, when dilution rates were 5 time, 10 times, and 40 times, biomass yields of algae were only 0.68, 0.82, and 0.59 g/L, respectively. Based on the discussion above, the optimum dilution rates of mother liquor, salt whey and liquid whey for algae growth were 20 times, 5 times and 5 times, respectively.

As shown in Figure 3.1, both low dilution rate and high dilution rate had negative effects on biomass yield of algae because low concentrations of nutrients were not enough to support algae growth while high concentrations of nutrients or metal elements may be toxic to algae growth on wastewater. Under the unfavorable conditions, self-protection

mechanism in algal cells was activated and the algae growth was prohibited (Stehfest et al., 2005).

Wastewater used for algae cultivation include municipal wastewater, agricultural waste effluent, and industrial waste stream (Chinnasamy et al., 2010; Lu et al., 2015; Zhou et al., 2011). In comparison with dairy processing wastewater, these wastewater reported by previous publications had higher biomass yields. For example, Zhou et al. (2011) grew *Chlorella* sp. on municipal wastewater, and produced 0.9 g/L dry algae biomass. In this chapter, the experimental results indicated that the nutrient profile of dairy processing wastewater was not well balanced in comparison with the artificial medium. For example, the concentration of NH_3-N was very low in diluted dairy processing wastewater. According to the previous studies, the exhaustion of one or more nutrients in wastewater or artificial medium would limit the algae growth even if other nutrients were still enough. Hence, it was supposed that the dilution of dairy processing wastewater reduced the concentrations of some nutrients and the nutrients deficiency became the limiting factor to algae growth.

3.3.2.2. Nutrients removal

To find out the limiting factors to algae growth, changes of NH_3-N, TN, TP, and COD in wastewater were analyzed (Figure 3.2). The result suggested that NH_3-N removal efficiencies in all three types of wastewater were 100% during the growth period. Algae in dairy processing wastewater consumed all NH_3-N on Day 2. The main reason for the fast consumption of NH_3-N is that the initial content of NH_3-N in diluted dairy wastewater was very low.

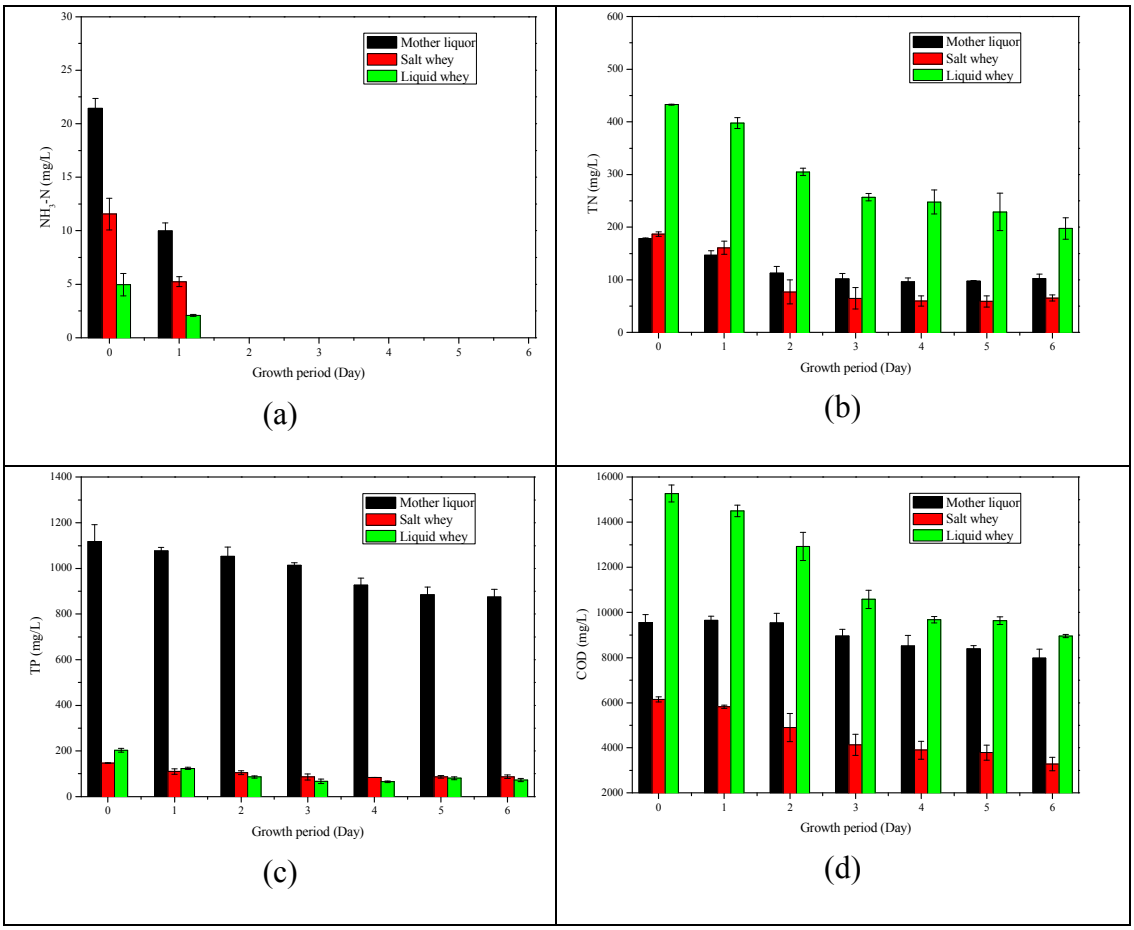


Figure 3.2. Nutrients removal in individual wastewater

The results in Figure 3.2 indicated that except for NH_3-N, concentrations of other nutrients, such as TN, COD and TP, were at very high levels. Therefore, the deficiency of NH_3-N should be the limiting factor to microalgae growth and nutrient removal in the dairy processing wastewater. In addition, the dairy processing wastewater with high concentrations of nutrients would cause very serious environmental pollution to the water body.

3.3.3. Biomass improvement by adding chemicals

In order to confirm the hypothesis proposed above, ammonium chloride was added into three types of dairy processing wastewater to increase the NH_3-N concentration. The growth of algae in dairy processing wastewater with ammonium chloride (Figure 3.3) indicated that the maximum biomass yields of algae in mother liquor, salt whey, and liquid whey were 3.24, 1.65, 2.34 g/L, respectively. Therefore, algae growth in dairy wastewater added with ammonium chloride was much better than the algae growth reported by previous studies. Based on the discussion above, it is an effective way to mitigate ammonia deficiency by increasing NH_3-N concentration in dairy processing wastewater. This result confirmed the hypothesis that ammonia deficiency was one of the barriers to algae growth in dairy processing wastewater.

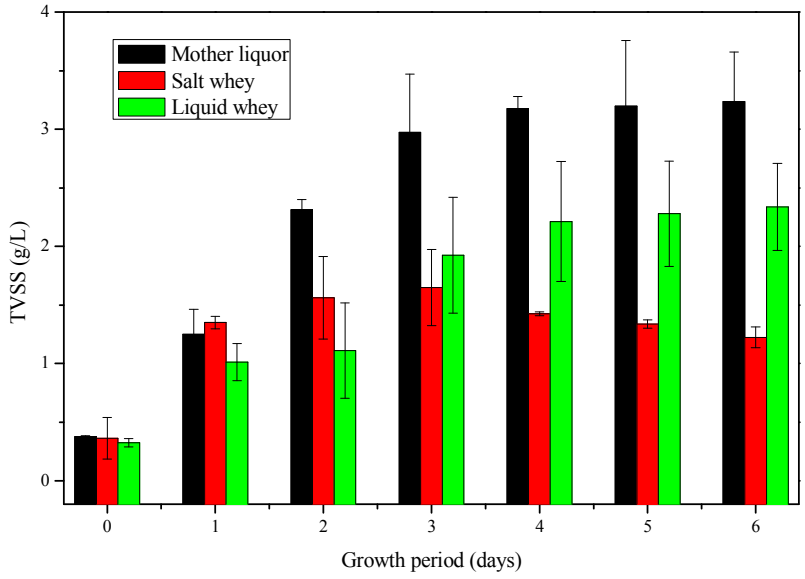


Figure 3.3. Growth of algae on dairy wastewater with NH_3-N addition

This result in Figure 3.3 is in agreement with some previous publications. For example, the densities of some algae in artificial medium and wastewater were doubled with the

addition of NH_3-N (Muller-Parker et al., 1994). The main reason for this phenomenon is that adding NH_3-N in medium or artificial medium provides sufficient nutrient to algae growth and replication. In the dairy processing wastewater added with ammonium chloride, because of the protein synthesis, algae had much active metabolism which contributed to the increase of biomass yield and nutrients removal.

3.3.4. Growth of algae in wastewater after mixing

Although adding artificial chemicals could increase the biomass yield of algae grown in dairy processing wastewater, it is hard to realize the wide application of this strategy in large scale system because the cost of artificial chemicals are very high. According to literature review, some food processing wastewater had very high concentration of NH_3-N. In this study, a meat processing wastewater which was obtained from a local slaughterhouse was mixed with the dairy processing wastewater by 1:1 (v/v). Nutrient profile of this meat processing wastewater included: TP, 97.1 mg/L, NH_3-N, 307.5 mg/L, TN, 416.0 mg/L, and COD, 7940 mg/L. In theory, adding this meat processing wastewater in dairy processing wastewater would increase the concentration of NH_3N and further promote algae growth. Nutrient profile of wastewater after mixing indicated that mixed wastewater had much higher concentration of NH_3-N.

3.3.4.1. Algae growth

Growth of algae wastewater after mixing (Figure 3.4) showed that the biomass yields of algae was the highest on Day 5. In addition, after mixing, the maximum biomass yields of

algae in mother liquor, salt whey, and liquid whey reached 2.66, 1.32, and 2.00 g/L, respectively. According to Table 4.3, the specific growth rates of algae in the dairy processing wastewater after mixing were 1.15, 1.59, and 2.93 day^{-1}, respectively, Therefore, it is an effective strategy to mitigate NH_3-N deficiency in dairy processing wastewater by mixing. In addition, after mixing, the biomass yield of algae increased.

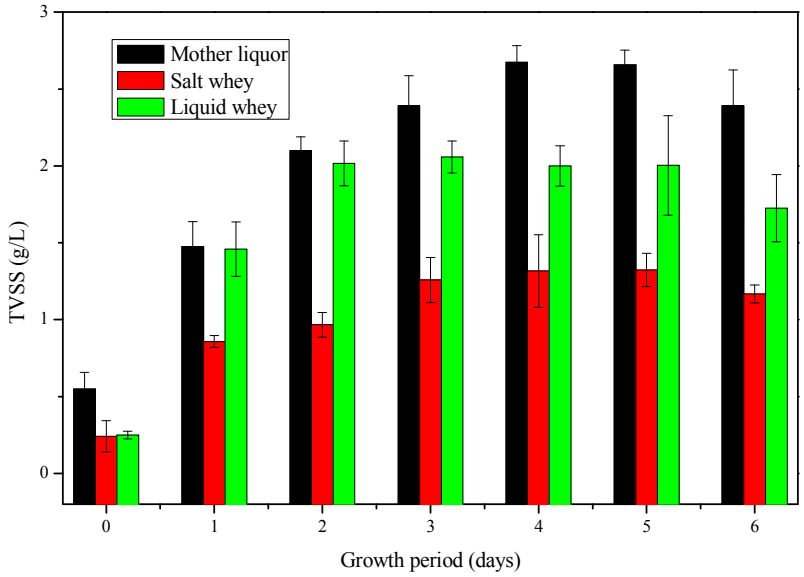


Figure 3.4. Growth of algae on mixed wastewater

In the dairy processing wastewater after mixing, salt whey had the lowest improvement rate. The main reason for this phenomenon is that in the wastewater of salt whey, there are many other limiting factors to algae growth. Therefore, the mitigation of NH_3-N deficiency may not be enough to support algae growth.

3.3.4.2. Nutrients removal

Nutrients removal in dairy processing wastewater after mixing (Figure 3.5) indicated that removal efficiencies of NH_3-N in mother liquor, salt whey, and liquid whey reached

100%, 92.14%, and 98.08%, respectively while TN removal in the dairy processing wastewater increased to 60.54%, 71.10% and 57.81%, respectively. Therefore, it is confirmed that the high concentration of NH_3-N in wastewater after mixing mitigate the barrier to algae growth and increased biomass yield.

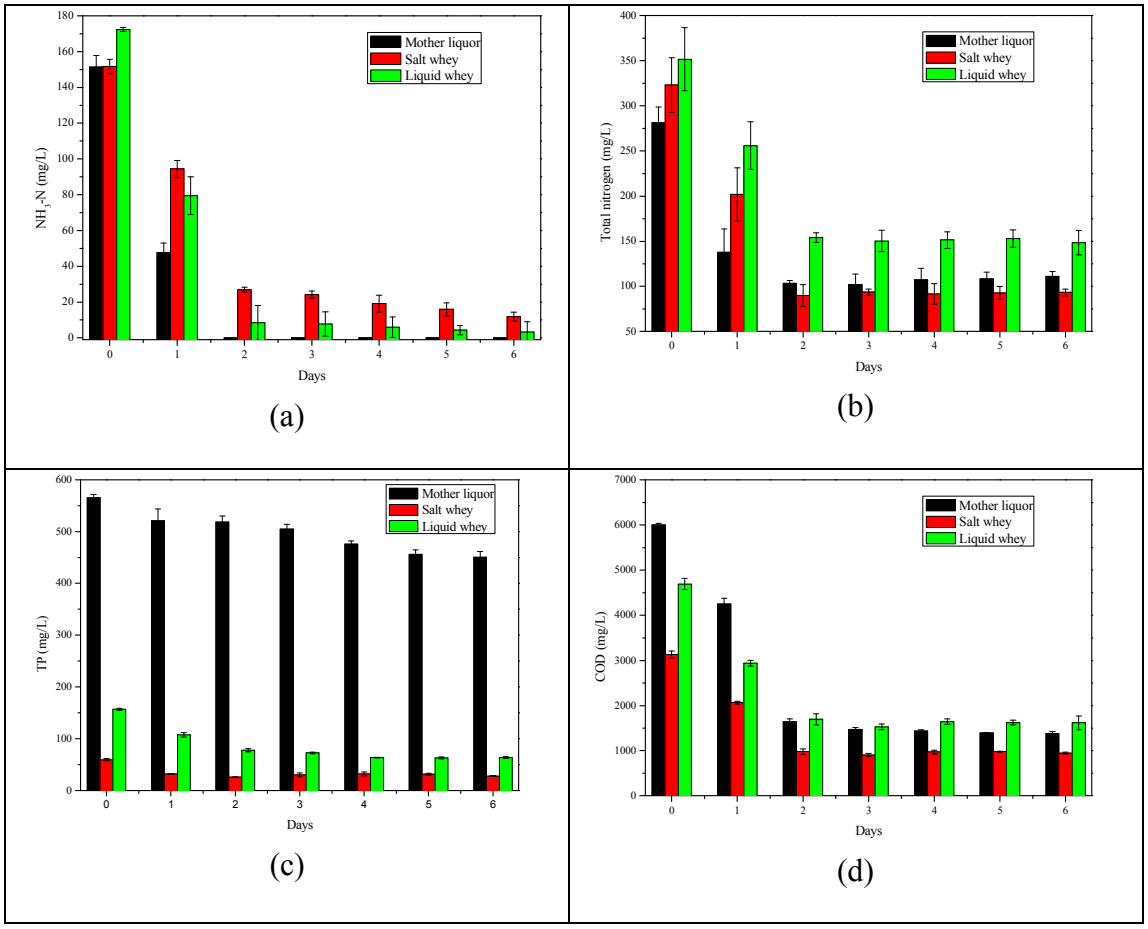


Figure 3.5. Nutrient removal efficiencies of mixed wastewater

As shown in Table 3.3., concentrations of TP in mother liquor (mixed), salt whey (mixed), and liquid whey (mixed) after algae cultivation were 450.7, 27.9, and 63.8 mg/L, respectively. After algae growth in wastewater after mixing, concentrations of COD left in mother liquor, salt whey, and liquid whey were 1377, 947, and 1619 mg/L, respectively (Table 3.3). Therefore, concentrations of TP and COD left in mixed dairy processing wastewater were reduced to a much lower level.

Table 3.3. Numerical values for the parameters of wastewater treatment

	K (g/L)	*a*	*r* (day^{-1})	R^2	TN (mg/L)	NH_3-N (mg/L)	TP (mg/L)	COD (mg/L)
Mother liquor	1.16	0.87	0.61	0.963	102.5	0	874.0	7980
Salt whey	0.97	0.82	1.06	0.987	65.5	0	87.0	3284
Liquid whey	0.95	0.85	0.76	0.967	197.5	0	72.6	8950
Mother liquor (mixed)	2.68	1.16	1.15	0.980	111.0	0	450.7	1377
Salt whey (mixed)	1.32	1.51	1.59	0.961	93.3	11.9	27.9	947
Liquid whey (mixed)	2.06	2.00	2.93	0.999	148.3	3.3	63.8	1619

Based on the discussion above, dairy wastewater mixed with meat processing wastewater with higher concentration of NH_3-N could promote algae growth and increase the nutrients removal efficiencies. Accordingly, the economic benefits of algae growth increased and the potential risks of environmental pollution could be effectively controlled. This chapter confirmed that wastewater mixing is a good strategy to for nutrients recycling and biomass production. To realize the commercialization of this strategy, food industries could set up pipelines for wastewater mixing before algae cultivation.

3.3.5. Composition of algae grown on dairy wastewater

As shown in Table 3.4, protein content of algae biomass harvested from the individual dairy processing wastewater without mixing was about 43.16-49.14% while the protein content of algae biomass harvested from wastewater after mixing reached 55.98%. Lipid content of algae biomass harvested from individual dairy wastewater without mixing was about 23.95-34.04% while the lipid content of algae biomass harvested from mixed wastewater was lower than 20.81%. The most possible reason for this phenomenon is that wastewater after mixing had higher concentration of NH_3-N which is an essential substrate for protein synthesis in microalgae (Gouveia & Oliveira, 2009). As a result, algae biomass harvested from this mixed wastewater could be used for the production of animal feed. In the practice, dilution rates and mixture ratios in the pretreatment of wastewater could be adjusted to change the nutrient composition of algae biomass.

Table 3.4. Composition of algae grown on dairy wastewater

	Protein (%)	Lipid (%)	Other * (%)
Mother liquor	49.14	28.74	22.12
Salt whey	43.89	34.04	22.07
Liquid whey	43.16	23.95	32.89
Mother liquor (mixed)	57.20	20.25	22.55
Salt whey (mixed)	55.98	20.81	23.21
Liquid whey (mixed)	66.91	19.10	13.99

* Other components include carbohydrates, nucleic acids, etc.

According to previous studies, the protein content of soybean could reach 33.1%. Therefore, the protein content of soybean is much lower than that of algae biomass harvested from the mixed dairy processing wastewater (Hymowitz et al., 1972; Wolf et al., 1982). In addition, biomass productivity of algae is much higher than the biomass productivity of most traditional crops (Chisti, 2007). Hence, algae cultivation on dairy processing wastewater could be a practical strategy to produce the protein at low cost. According to the compositions of algae biomass, the biomass harvested from the dairy processing wastewater after mixing pretreatment can be utilized to produce both protein-based products, such as animal feed.

3.4. Conclusions

The conclusions of this chapter include: (1) One of the factors limiting algae cultivation in dairy processing wastewater was the deficiency of NH_3-N; (2) Wastewater mixing is an effective way to promote algae growth in the dairy processing wastewater; (3) Algae biomass harvested from the wastewater had high protein content (55.98%-66.91%). So the biomass could be utilized to produce protein-based products, such as animal feed; and (5) In the future, more effects are needed to realize the commercialization of this strategy.

Chapter 4. Use of meat processing wastewater for algae culture

4.1. Introduction

One of the main concerns with using wastewater for microalgae biomass production is the nutrient profile of the wastewater. For example, most wastewater may not have balanced nutrient profiles which are necessary to support algae growth. Zhou et al. (2011) indicated that in municipal wastewater, the organic carbon is not enough to support algae growth. The nutrient deficiency not only limits the algae growth, but also reduce the nutrient removal efficiencies. In addition, some wastewater may contain toxic compounds which would prohibit algae growth (Hughes & Poole, 1991). The toxic compounds could also be absorbed by microalgae cells. As a result, the harvested algae biomass will not be utilized for animal or human consumption. Since food processing wastewater contain few toxic compounds (Jacobsen et al., 2013), they may be exploited to produce algae biomass for animal feed or food uses.

Growth of microalgae in food processing wastewater has been widely reported in the publications (Blier et al., 1995; Kern & Idler, 1999). The strategy of using food processing wastewater for microalgae cultivation has not been successfully commercialized. One of the main reasons is that the imbalanced nutrient profile of wastewater could limit the algae growth and reduce the biomass yield. For example, in the research of Kern & Idler (1999), meat processing wastewater had low concentrations of nutrients, including 15.0 mg/L TP and 125.0 mg/L TN (Kern & Idler, 1999). To solve the problems caused by unbalanced nutrient profile in wastewater, previous publications applied anaerobic digestion or acid digestion to convert solid particles in wastewater to

soluble nutrients or added artificial chemicals into wastewater (Wang et al., 2013c). Due to the high operation cost, however, these strategies have not been wide used.

In this chapter, the wastewater was obtained from meat processing industry, which is one of the major food industries in Minnesota. A meat processing factory could produce more than 10,000,000 L waste stream daily (Bhamidimarri, 1991). Previous publications indicated that meat processing wastewater have the essential nutrients for algae growth (Thayalakumaran et al., 2003) but the concentrations of some nutrients are very low, not sufficient to support algae growth. According to the literature review, there was not much research on the use of microalgae in the nutrients recovery from meat processing wastewater.

In this chapter, the wastewater obtained from in a meat processing plant in Minnesota was exploited to grow algae. The main purposes of this chapter include finding out the barriers to algae growth in meat processing wastewater and developing a practical method to promote microalgae growth. The main objectives of this chapter include (1) measuring the nutrient profile and metal profile of different types of meat processing wastewater; (2) analyzing the growth of microalgae in non-mixed meat processing wastewater and mixed meat processing wastewater and testing the nutrient removal efficiencies by microalgae; (3) measuring the nutrient compositions in microalgae at different growth conditions.

4.2. Materials and methods

4.2.1. Materials and chemicals

Five types of meat processing wastewater, including REFINERY, KILL, MPGP, CUT, and DS, were obtained from local meat processing factory in Minnesota, USA. The wastewater was centrifuged for 10 min at 8000 RPM to remove suspended solid particles and autoclaved for 30 min at 121°C. In the practice, the separated solids from wastewater could be exploited to produce fertilizer. The artificial medium, which was used as a reference for comparison purpose, contained: H_3BO_3 (11 mg/L), $MgSO_4.7H_2O$ (0.1 g/L), $CoCl_2.6\ H_2O$ (1.5 mg/L), K_2HPO_4 (0.11 g/L), $FeSO_4.7H_2O$ (5 mg/L), $CaCl_2.2H_2O$ (0.05 g/L), $ZnSO_4.7H_2O$ (22 mg/L), Tris (2.42g/L), NH_4Cl (0.375 g/L), KH_2PO_4 (0.06 g/L), $CuSO_4.5\ H_2O$ (1.5 mg/L), $(NH_4)_6Mo_7O_{24}.4H_2O$ (1 mg/L), $MnCl_2.4H_2O$ (5 mg/L), and acetic acid (1 mL/L).

4.2.2. Growth and chemical analysis

4.2.2.1. Determination of algal growth

In this chapter, biomass yield was measured daily according to the previously published method (Zhou et al., 2012b). The growth rate of microalgae was calculated according to Equation 1.

$$R = (W_t - W_0)/(T_t - T_0) \quad \text{Eq. 1}$$

where R is the growth rate of microalgae based on TVSS; T_0 and T_t are time on day 0 an day t; W_t and W_0 are the TVSS at day t and day 0, respectively.

To assess the effects of wastewater mixing on biomass production and nutrient removal, the theoretical average value and nutrient removal were calculated. Equation 2 was applied for the calculation of theoretical average value of biomass yield.

$$T_b = (X_1 + X_2)/2 \quad \text{Eq. 2}$$

where T_b is the theoretical average biomass yield in the mixture of wastewater; X_1 and X_2 are the biomass yield on individual wastewater.

Theoretical average value of nutrient removal efficiency was calculated according to Eq. 3.

$$T_n = (N_1 \times RE_1 + N_2 \times RE_2)/(N_1 + N_2) \quad \text{Eq. 3}$$

where T_n (%) is the theoretical average nutrient removal efficiency; N_1 and N_2 are concentrations of certain nutrient in individual wastewater; RE_1 and RE_2 are nutrient removal efficiencies (%) in individual wastewater.

4.2.2.2. Nutrient analysis

Various nutrients, including NH_3-N, TN, COD, and TP were measured by using a spectrophotometer according to the previously published method (Li et al., 2011b). Concentrations of nutrients in wastewater were expressed as mg/L.

4.2.2.3. Analysis of composition in microalgae biomass

Protein content in microalgae biomass harvested from wastewater was measured by using CE-440 elemental analyzer (Exeter Analytical Inc., Chelmsford, MA) according to the

previously published method (Hu et al., 2013). Conversion ratio of nitrogen-to-protein for the calculation of protein content was 6.25 (Dominguez, 2013).

Harvested algae biomass was dried in vacuum dryer before the conduction of oil extraction. Total lipid in microalgae biomass was analyzed according to the one-step extraction method described by Folch et al. (Folch et al., 1957). About 40 mg dried algae biomass was mixed with 2:1 chloroform/methanol (v/v) solution. After oil extraction process, the organic solvent was evaporated by using Nitrogen Evaporator (Organomation Associates, Inc., USA). After evaporation, the lipid left at the bottom of tube was weighted.

4.2.3. Treatment of solids in wastewater by acid hydrolysis

The suspended solids in wastewater could not be utilized by microalgae cells. In addition, these suspended solids could also reduce the light transmission and create an unfavorable condition to algae growth. As a result, the photosynthesis in microalgae cells would be limited. In the lab scale experiment, the most efficient way to separate the solids from liquid is centrifugation. However, centrifugation is energy intensive and not practical in the practice. In this chapter, acid hydrolysis was conducted to reduce the concentration of suspended solids in meat processing wastewater. According to the results of preliminary experiment, the optimum ratio of liquid to solid is 3:1 (v/v) for the acid hydrolysis. In this chapter, the hydrolysis parameters, such as hydrolysis temperature, acid concentration, and time, were studied (Table 4.1). The nutrients profile of meat processing wastewater was measured after acid hydrolysis.

Table 4.1. Design of single factor experiment for acid hydrolysis

	A	B	C
Acid concentration (%)	a	4	4
Temperature (^{o}C)	95	b	95
Time (h)	10	10	c
Variables	0	55	0
	4	65	5
	8	75	10
	12	85	15
	16	95	20

4.3. Results and discussion

4.3.1. Nutrient and metal profiles of wastewater

Concentrations of four nutrients, including TN, TP, NH_3-N, and COD, in meat processing wastewater, were measured. As shown in Table 4.2, compared with the artificial medium, the five types of meat processing wastewater were insufficient in some nutrients. For example, other four types of wastewater, except KILL, did not contain sufficient TN, NH_3-N, and COD, compared with TAP medium. In this study, the difference of nutrient profiles was mainly caused by the different meat processing steps. Previous studies reported that some meat processing wastewater had high concentration of COD (1500-

11118 mg/L) while the concentration of TP in the wastewater was even lower than 20 mg/L (Johns, 1995; Sayed & de Zeeuw, 1988). Therefore, the deficiency of one or more nutrients is a very critical issue in the treatment of meat processing wastewater.

Table 4.2. Nutrient profiles of meat processing wastewater

Wastewater	TN (mg/L)	TP (mg/L)	NH_3-N (mg/L)	COD (mg/L)
DS	76.5	10.2	11.1	734
CUT	64.8	45.9	8.2	1019
KILL	327.6	46.8	193.0	3560
REFINARY	117.5	5.6	101.7	1016
MPGP	91.3	32.9	2.2	2035
TAP medium	364.4	28.6	132.0	3870
DS+KILL	204.9	16.3	92.5	2100
CUT+KILL	212.0	29.7	102.1	2100
REFINARY+KILL	251.0	16.4	169.6	2340
MPGP+KILL	197.6	22.8	101.6	3020

"+" means the mixture of the wastewater

Some metal elements, such as calcium, zinc, manganese, copper, etc., are essential to the microalgae growth while some metal elements, such as lead and aluminum, have negative effects on the microalgae growth (Gadd & Griffiths, 1977; Hughes & Poole, 1991). It has been proven that the deficiency of essential metal element or the high concentrations of toxic metal elements would limit the microalgae growth or even cause the failure of

microalgae cultivation. Besides, some toxic metal elements in aqueous phase could be absorbed by microalgae. Accordingly, the microalgae biomass would not be suitable for the production of animal feed or food ingredients (Bulgariu & Bulgariu, 2012). As shown in Table 4.3, meat processing wastewater had the essential elements to microalgae growth. However, the concentrations of some macro metal elements, such as Mg, Ca, and Na, in meat processing wastewater were much higher than those in artificial medium. According to the metal profile analysis, meat processing wastewater could be used for algae production.

Table 4.3. Metal profiles of meat processing wastewater

Metal concentration (mg/L)	DS	CUT	KILL	REFINERY	MPGP	TAP medium
B	0.10	0.06	0.05	0.08	0.06	2.02
Ca	22.58	17.10	13.47	13.23	61.26	13.60
Co	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.40
Cu	0.79	< 0.02	0.03	< 0.02	< 0.02	0.40
Fe	0.21	0.39	0.48	0.37	1.26	1.00
Mg	7.96	18.63	19.91	20.82	20.25	9.76
Mn	0.03	0.01	0.01	0.01	0.02	1.41
Mo	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.60
Na	136.30	100.80	55.11	199.20	238.80	6.18
Zn	0.14	0.09	0.03	0.06	1.10	4.93

In this study, the pH value of MPGP was about 3.4 while the pH values of other four types of meat processing wastewater were neutral. Therefore, the wastewater of MPGP was subjected to pH adjustment before algae cultivation.

4.3.2. Growth of algae on individual wastewater

4.3.2.1. Microalgae growth

As shown in Figure 4.1, on Day 6, biomass yield of microalgae grown in KILL, MPGP, CUT, and REFINERY reached 1.800, 0.675, 0.642, and 0.633 g/L, respectively. However, the microalgae in the wastewater of DS did not have any growth. The main reason for the failure of algae cultivation in the wastewater of DS is that the high concentrations of toxic metal elements, such as copper, caused the death of microalgae cells. It was reported that if the concentration of copper is higher than 0.5 mg/L, the growth of *Chlorella* sp. would be limited (Wong & Chang, 1991). The concentration of copper in the wastewater of DS was about 0.79 mg/L. Furthermore, because of the low concentrations of essential nutrients, the wastewater of DS was not favorable to microalgae growth. Compared with artificial medium, the meat processing wastewater, except KILL, were not suitable to microalgae cultivation and biomass production. According to the growth curve of microalgae, the growth period of microalgae in the meat processing wastewater should be 6-day. In terms of biomass yield, the wastewater of KILL was the most favorable wastewater for the production of microalgae biomass. The lack of one or two nutrients in wastewater became a barrier to microalgae cultivation in other four types of meat processing wastewater.

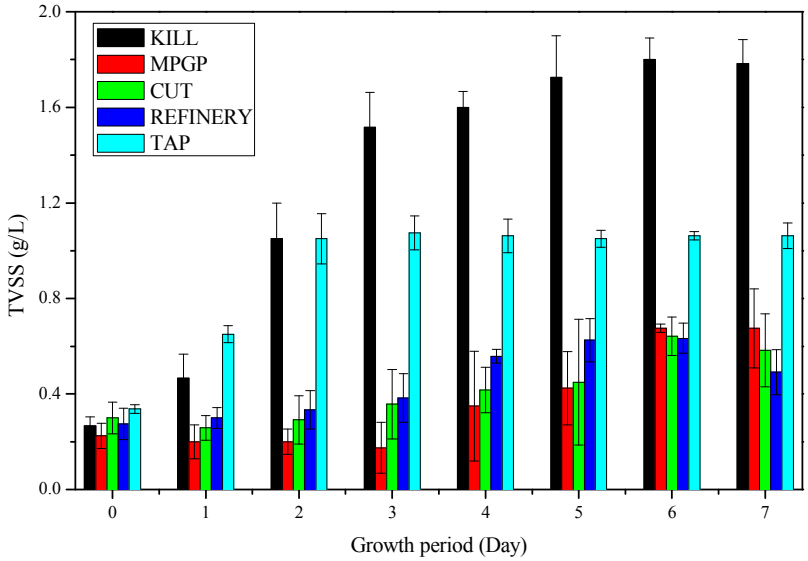


Figure 4.1. Growth curve of algae cultivated on non-mixed wastewater

4.3.2.2. Nutrients removal

Figure 4.2 is about the nutrients removal in non-mixed meat processing wastewater during microalgae cultivation. In the wastewater of DS, since microalgae did not have any growth, the nutrients removal efficiency was 0%. As shown in Figure 4.2 (a), NH_3-N removal efficiencies in the wastewater of KILL, MPGP, CUT and REFINERY reached 45.60%, 100.00%, 100.00% and 60.50%, respectively. In addition, the removal efficiencies of TN in the wastewater of KILL, MPGP, CUT and REFINERY reached 33.29%, 33.21%, 0%, and 32.74%, respectively. As shown in Figure 4.2(c), the removal efficiencies of TP in the wastewater of KILL, MPGP, CUT and REFINERY reached 65.37%, 30.85%, 0%, and 100.00%, respectively. The removal efficiencies of COD were shown in Figure 4.2 (d).

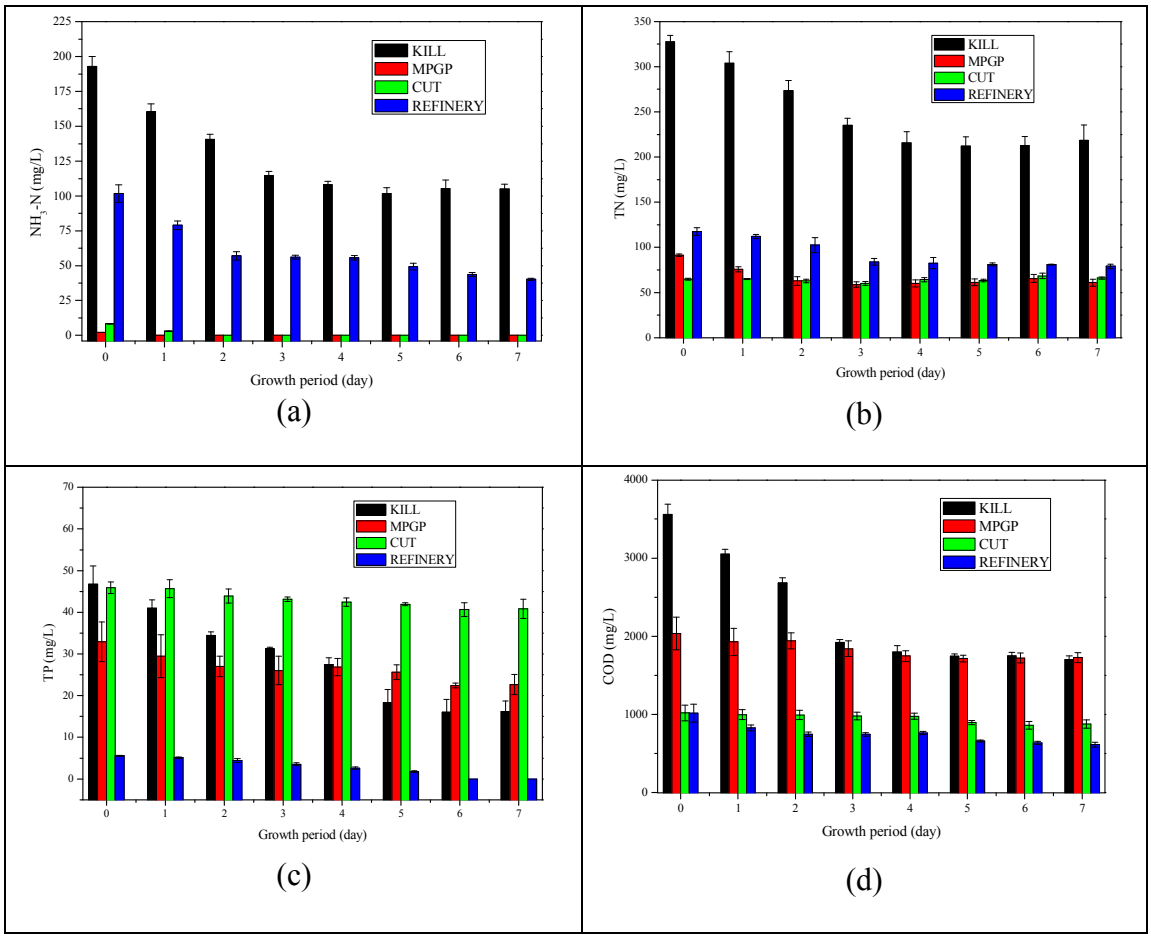


Figure 4.2. Removal of nutrients in non-mixed wastewater

There are many possible reasons for the difference in nutrient removal and biomass production in five types of meat processing wastewater. For example, in the meat processing wastewater, some nutrients may not be utilized by microalgae cells in an efficient way. In this study, it was hypothesized that the organic carbon in the wastewater of CUT could not be utilized by microalgae cells efficiently. Since microalgae cells could not absorb the large solid particles directly, nutrients in the form of large particles in wastewater could not be absorbed by microalgae cells (Zemke-White et al., 2000). The second possible reason is that the deficiency of one or more nutrients in the wastewater or culture medium would prohibit the increase of biomass yield. Accordingly, the removal of other nutrients would be reduced to a lower level. The last possible reason is that in

some meat processing wastewater, microalgae growth was limited by the high concentration of toxic metal elements (Kong & Chen, 1995).

4.3.3. Growth of algae on mixed wastewater

4.3.3.1. Biomass yields of algae

According to the previous experimental results, in this study, the wastewater of KILL was good for microalgae cultivation. In theory, the addition of KILL into other meat processing wastewater would mitigate the nutrients deficiency and increase the biomass yield. In this study, the wastewater of KILL was mixed with other four types of wastewater by 1:1 (v/v) individually. As shown in Table 4.4, nutrient profiles of mixed meat processing wastewater were very similar with the nutrient profiles of artificial medium.

Table 4.4. Comparison of algae growth on non-mixed and mixed wastewater

Wastewater	TVSS (g/L)	TN (%)	TP (%)	NH_3-N (%)	COD (%)
KILL+CUT	1.538	50.94	44.95	90.38	29.52
KILL and CUT	1.221	27.79	33.06	45.77	40.62
KILL+REFINERY	1.400	49.48	54.45	68.75	43.91
KILL and REFINERY	1.216	35.39	69.07	50.24	49.45
KILL+MPGP	1.388	30.06	63.51	87.43	7.95
KILL and MPGP	1.237	33.27	51.26	44.30	38.73
KILL+DS	0.675	44.46	52.11	82.40	3.21
KILL and DS	0.900	26.99	53.67	41.88	4.32

As shown in Figure 4.3, biomass yields of microalgae grown in CUT+KILL, REFINERY+KILL, MPGP+KILL, and DS+KILL reached 1.538, 1.400, 1.388, and 0.675 g/L, respectively. Biomass yields on CUT+KILL, REFINERY+KILL, and MPGP+KILL were much higher than the biomass yield of microalgae grown in artificial medium. In addition, the mixed meat processing wastewater produced much more biomass than the non-mixed meat processing wastewater. For example, the mixture of the wastewater of DS and KILL promoted the microalgae growth while the non-mixed DS could not support microalgae growth.

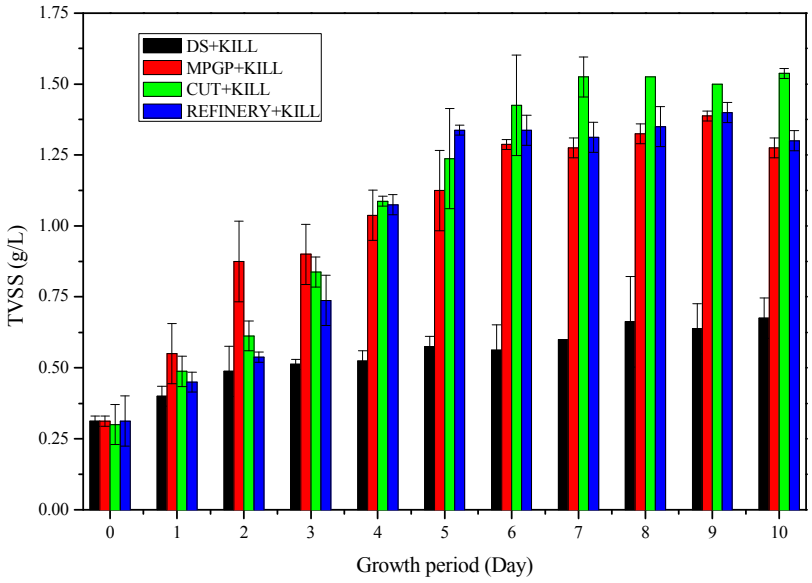


Figure 4.3. Growth curve of algae cultivated on mixed wastewater

Data in Table 4.4 indicated that other three types of mixed meat processing wastewater, except KILL+DS, had much higher biomass yield. Compared with the theoretical average biomass yields, biomass yields of microalgae grown in the mixed wastewater of KILL+CUT, KILL+REFINERY, and KILL+MPGP increased by 25.96%, 15.13%, and 12.21%, respectively. Since the deficiency of one or more nutrients in meat processing wastewater was the barrier to microalgae cultivation in this study. The synergetic effects of different types of meat processing wastewater in the mixed system successfully modified the nutrient profile and promoted the microalgae growth.

4.3.3.2. Nutrients removal

As shown in Figure 4.4, removal efficiencies of TN in the mixed wastewater of KILL+CUT, KILL+REFINERY, and KILL+DS increased a lot. In addition, removal efficiencies of NH_3-N in all of the mixed meat processing wastewater were much higher

compared with the theoretical average value of nutrient removal. For example, NH_3-N removal efficiency in the mixed wastewater of KILL+CUT was about 87.43% while the theoretical average value was only about 44.30%. Previous publications suggested that in some wastewater, the essential nutrients are in the form of macromolecular materials which could not be directly absorbed by microalgae cells (Stehfest et al., 2005). In this study, the microalgae in the wastewater of CUT, REFINERY, DS and MPGP were in a condition without sufficient nitrogen. After wastewater mixing pretreatment, the concentration of nitrogen in the wastewater increased significantly and the nitrogen deficiency was mitigated. As a result, biomass yield and nutrients removal efficiencies in the mixed meat processing wastewater were much higher than the biomass yield and nutrients removal efficiencies in non-mixed wastewater.

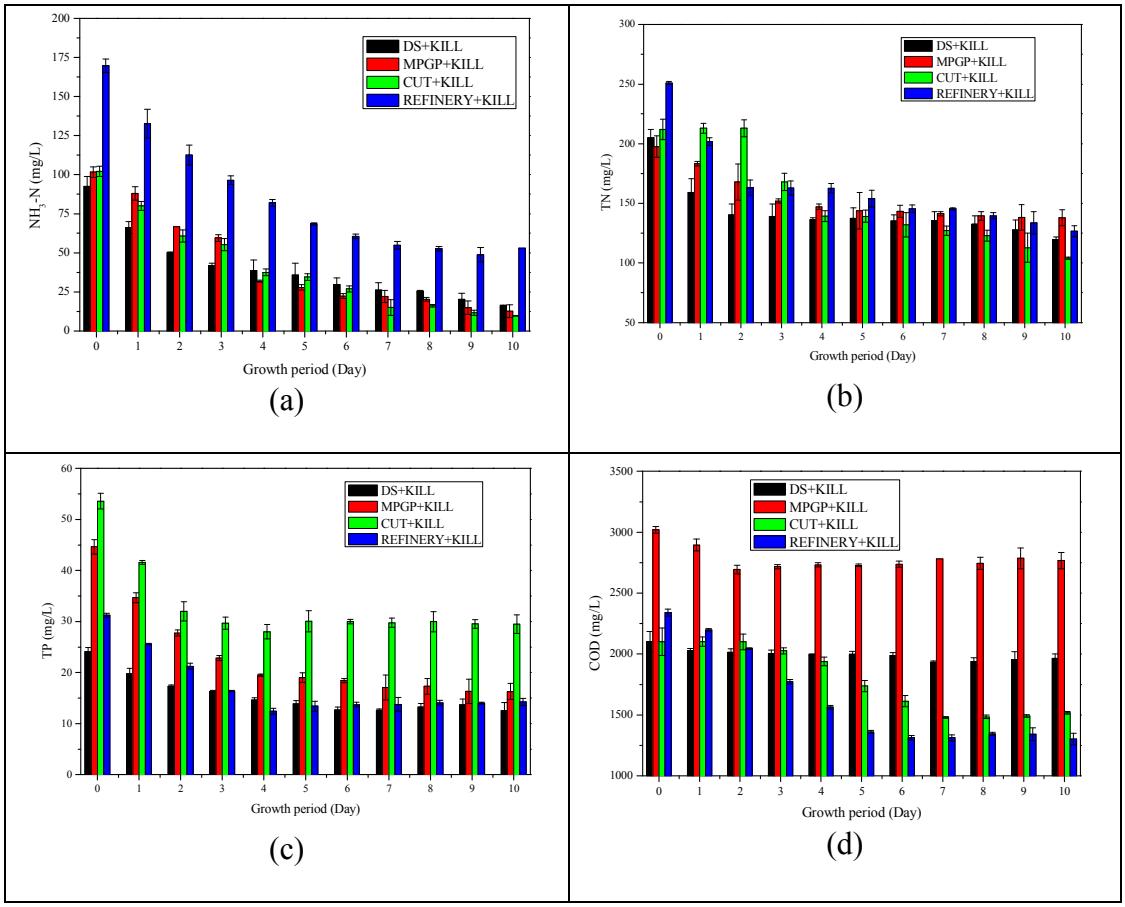


Figure 4.4. Removal of nutrients in mixed wastewater

However, the removal efficiency of COD in the mixed meat processing wastewater was much lower than the theoretical value. For example, removal efficiency of COD in the mixed wastewater of KILL+CUT was only about 29.52%, much lower than the theoretical value. Since microalgae grown in wastewater with low nitrogen concentration are exposed to unfavorable conditions and are prone to synthesize lipid for survival (Siaut et al., 2011). In this chapter, the concentration of nitrogen in some non-mixed wastewater was not sufficient to support microalgae growth, so lipid synthesis in microalgae cells was significantly enhanced (Rodolfi et al., 2009). Under this condition, microalgae were prone to utilize organic carbon in meat processing wastewater for the lipid synthesis. However, in the mixed wastewater, concentration of nitrogen for microalgae growth increased with the addition of KILL. Accordingly, microalgae could have sufficient nitrogen for protein synthesis while the lipid synthesis was limited. As a result, the removal efficiency of COD in mixed wastewater was much lower than that in non-mixed wastewater.

4.3.4. Composition of microalgae biomass harvested from wastewater

As shown in Table 4.5, protein contents of the algae biomass harvested from CUT, REFINERY and MPGP were about 51.58-60.33%, which falls in the range of protein contents in *Chlorella* sp. reported in previous studies (Hymowitz et al., 1972; Wolf et al., 1982). It was also discovered that the microalgae grown in the mixed meat processing wastewater contained much more protein (60.87-68.65%). The difference in protein content is attributed by the concentration of nitrogen in both mixed and non-mixed

wastewater. Theoretically, wastewater with low concentration of nitrogen would limit the protein synthesis in microalgae cells (Wang et al., 2009) but promote the lipid synthesis (Scott et al., 2010). As a result, protein content of microalgae in mixed meat processing wastewater was much higher than the protein content of microalgae harvested from non-mixed wastewater, including CUT, MPGP, and REFIERY.

Table 4.5. Composition of algae grown on various meat processing waste streams

Wastewater	Lipid (%)	Protein (%)	Other* (%)
KILL+CUT	17.54	68.65	13.81
KILL+REFINERY	20.57	64.76	14.67
KILL+MPGP	18.89	61.20	19.91
KILL+DS	14.50	60.87	24.63
CUT	21.01	51.58	27.41
REFINERY	23.95	60.33	15.72
MPGP	25.60	55.08	19.32
KILL	15.87	63.31	20.82

* Other components include carbohydrates, nucleic acids, etc.

Compared with soybean, microalgae have some advantages in the production of protein. First, the protein content of microalgae harvested from wastewater or culture medium could be much higher than the protein content of soybean (33.1% to 49.2%) (Hymowitz et al., 1972; Wolf et al., 1982). In addition, the oil content (17.88%) in algae is similar with the oil content (18%) in soybean (Mata et al., 2010). Last but not the last, oil yield of

microalgae could reach 136900 L/ha year while the oil yield of soybean could only reach 446 L/ha year (Chisti, 2007). Therefore, to produce protein and oil, microalgae cultivation is better than the soybean cultivation. Algae cultivation conducted in mixed meat processing wastewater without toxic contaminants could be a valuable resource in the industry.

4.3.5. Acid hydrolysis of solids in wastewater

4.3.5.1. Content of solids in wastewater

In the experiment, a lot of suspended solids were observed in the wastewater of KILL. In algae cultivation, the suspended solids will limit the light transmission and reduce the photosynthesis efficiency. To solve such a problem, the suspended solids were separated from the wastewater by centrifugation. In the large scale system, to reduce the cost, the separation of solids and wastewater was achieved by sedimentation. In this work, about 3.4 mL solids were obtained from 50 mL wastewater by centrifugation at 8000 rpm. Therefore, content of solid in wastewater was about 6.8% by volume.

4.3.5.2. Assessment of hydrolysis conditions

Three parameters, including acid concentration, hydrolysis temperature, and hydrolysis time, were assessed. As shown in Figure 4.5(a), with the increase of acid concentration, concentrations of soluble nutrients increased gradually. When the acid concentration was higher than 8%, the concentrations of soluble nutrients did not increase significantly. It was reported that sulfuric acid at high concentrations may cause the carbonization of

organics. So to control the use of acid and prevent potential carbonization, in this work, the concentration of acid was set as 8%. Besides the acid concentration, the extension of hydrolysis time also increased the concentrations of soluble nutrients. According to Figure 4.5(b), the conversion of solids to soluble nutrients mainly occurred in the first 15 hours. Since the hydrolysis has high requirement on the temperature maintenance, longer time will consume more energy. Therefore, the hydrolysis period should be controlled around 15 hours. As shown in Figure 4.5(c), temperature is another important factor that determines the hydrolysis of solids in wastewater. The results showed that high temperature could accelerate the hydrolysis process.

After hydrolysis, a large quantity of soluble nutrient was produced. From example, the concentration of COD had significant increase. In some conditions, after hydrolysis, the concentration of COD reached 10000 mg/L. In the practice, the medium with hydrolyzed solids from wastewater is a good resource for the cultivation of algae biomass with high oil content.

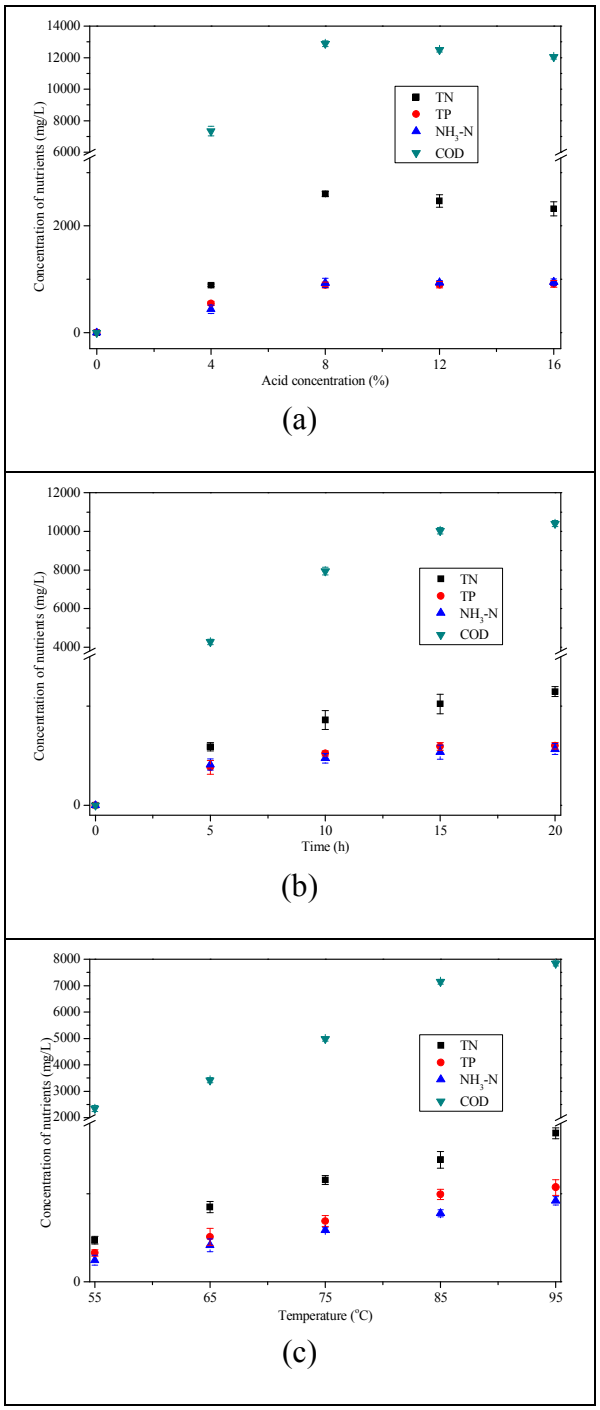


Figure 4.5. Assessment of hydrolysis conditions

4.4. Conclusions

The conclusions of this chapter include: (1) The deficiency of one or more nutrients should be a barrier to microalgae cultivation in non-mixed meat processing wastewater, except KILL; (2) Mixed wastewater could promote the microalgae growth; (3) It is an economic and efficient way to balance the nutrient profile and improve algae growth by mixing the wastewater; (4) Acid hydrolysis under optimized conditions could convert solids to soluble nutrients.

Chapter 5. Carbon-dependent alleviation of ammonia toxicity

5.1. Introduction

Many types of waste effluents have very high concentrations of NH_3-N. Therefore, the toxicity caused by high concentrations of NH_3-N to microalgae cultivation has been regarded as a barrier to microalgae-based wastewater treatment. In previous publications, the concentrations of NH_3-N in wastewater were reduced before algae growth (Park & Kim, 2015; Serna-Maza et al., 2014). Common pretreatment strategies for the reduction of concentration of NH_3-N removal include ammonia stripping and dilution (Lu et al., 2016). However, these strategies are not practical in the pilot scale system for microalgae-based wastewater treatment because of some technical disadvantages (Guštin & Marinšek-Logar, 2011). Therefore, ammonia toxicity to algae growth is still a serious problem for the sustainable development of microalgae-based wastewater treatment.

In algal metabolisms, NH_3-N is combined with alpha-ketoglutarate (α-KG) for the synthesis of glutamate. In microalgae cells, α-KG is a product of Krebs cycle (Zuñiga et al., 2016). The glutamate is synthesized through glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway. In microalgae cells, to alleviate ammonia toxicity, there should be enough α-KG.in microalgae cells. Optimization of the ratio of carbon to nitrogen is a good way to promote NH_3-N assimilation and alleviate ammonia toxicity in microalgae cells (Garcia et al., 2011). Previous studies suggested that the concentration of carbon source is important to NH_3-N assimilation (Magalhaes et al., 1992). For example, Magalhaes et al. (1992) reported that in specific conditions, NH_3-N assimilation in cells could be accelerated by the addition of α-KG.

According to the literature review, some questions should be answered: (1) Is it a possible strategy to alleviate ammonia toxicity by adding α-KG? (2) What are the effects of different carbon sources on ammonia assimilation in microalgae cells? This chapter was to answer these questions and propose a practical way to mitigate ammonia toxicity.

5.2. Materials and methods

5.2.1. Artificial wastewater and algal strain

The nutrients in artificial wastewater for the experiment include: KH_2PO_4 (0.054 g/L), $MgSO_4 \cdot 7H_2O$ (0.16 g/L), $CaCl_2 \cdot 2H_2O$ (0.08 g/L), K_2HPO_4 (0.108 g/L), and the trace element solution (1 mL/L). NH_4Cl was added into the artificial wastewater as the ammonia source to support microalgae growth. The initial pH value of the artificial wastewater was about 6.0 before microalgae inoculation. Artificial wastewater was autoclaved for 30 min at 121 ^oC to kill all the bacteria. In this chapter, both inorganic carbon (sodium bicarbonate) and organic carbon (glucose and citric acid) were used as carbon source for microalgae growth.

In this study, the microalgae strain used for the experiment is *Chlorella* sp. The microalgae were cultivated on TAP medium-based agar plate at room temperature (25±1 ^oC) before the formal experiment (Lu et al., 2015).

5.2.2. Algae growth and nutrients analysis

5.2.2.1. Measurement of algae growth

Cell density (cells/mL) and viability of microalgae cells (%) were analyzed to evaluate the growth of microalgae. Viability is a parameter to reflect the percentage of living microalgae cells in the total cells. In this study, the initial density of microalgae cells was about 5.0×10^6 cells/mL.

5.2.2.2. Measurement of nutrients

Assay kits for the measurement of ammonia (NH_3-N) and chemical oxygen demand (COD) were purchased from Hach (USA). The measurement was conducted according to the method reported by Lu et al. (2015).

5.2.2.3. Parameters calculation

Removal efficiency of NH_3-N was calculated according to Eq. 1.

$$\mathrm{NH_3-N\ removal\ efficiency\ (\%)} = \frac{A_0-A_t}{A_0}\times 100\% \quad \text{Eq. 1}$$

where A_0 and A_t are NH_3-N concentrations (mg/L) on Day 0 and Day t, respectively.

Theoretical NH_3-N removal and experimental NH_3-N removal (mg/L) were calculated based on Eq. 2 and Eq. 3, respectively.

$$\mathrm{Theoretical\ NH_3-N\ assimilation} = (K_t - K_0)\times N\times 14 \quad \text{Eq. 2}$$

$$\mathrm{Experimental\ NH_3-N\ assimilation} = A_0 - A_t \quad \text{Eq. 3}$$

where K_t and K_0 are concentrations of carbon source (mol/L) on Day t and Day 0. N is the conversion ratio of α-KG to certain carbon. According to the Krebs cycle, conversion

ratio of citric acid to α-KG is 1:1 (Huo et al., 2011). A_0 and A_t are NH_3-N concentrations (mg/L) on Day 0 and Day t.

5.2.3. Effects of exogenous carbon on NH_3-N assimilation

In this chapter, α-ketoglutaric acid disodium salt was added into artificial wastewater (with 2 g/L NH_4Cl) to promote NH_3-N removal and mitigate ammonia toxicity.

To reduce the cost of ammonia toxicity mitigation, three common carbon sources, sodium bicarbonate, citric acid, and glucose, were added into artificial wastewater at different concentrations. The carbon sources, including citric acid, sodium bicarbonate, and glucose, were selected because of two reasons. First, these three carbon sources could be obtained at very low cost. Second, glucose and citric acid could be utilized by microalgae cells directly through glycolysis and Krebs cycle. Some other organic carbon, such as acetic acid, should be converted into acetyl-CoA for further metabolic utilization (Castaño-Cerezo et al., 2009). To find out the principles applied to most microalgae species, glucose and citric acid were used by this chapter.

5.3. Results

5.3.1. Threshold of ammonia toxicity

As shown in Table 5.1, when the concentration of NH_3-N was 28.03 mM, the average viability of microalgae cells was about 80.6%. High concentrations of NH_3-N in artificial wastewater increased the percentage of dead microalgae cells. It was also observed that the maximum cell density reached 0.99×10^7 cells/mL when the concentration of NH_3-N

was 18.69 mM. However, when the concentration of NH_3-N was higher than 18.69 mM, maximum cell density started to decrease. Therefore, in certain range, the increase of NH_3-N concentration could promote microalgae growth and increase the average viability of cells. But when the concentration of NH_3-N was higher than the threshold, ammonia toxicity will limit the microalgae growth and reduce the survival efficiency of cells.

Table 5.1. Effects of NH_3-N on maximum cell density and average viability

Concentration of NH_4Cl (g/L)	Concentration of ammonia (mM)	Maximum cell density (10^7 cells/mL)	Average viability (%)
0	0	0.62	86.8±2.3
0.25	4.67	0.75	95.4±1.9
0.50	9.34	0.84	95.7±1.0
0.75	14.01	0.90	92.6±2.8
1.00	18.69	0.99	90.4±2.7
1.25	23.36	0.88	84.6±3.9
1.50	28.03	0.84	80.6±3.9
2.00	37.38	0.79	69.9±5.6
4.00	74.77	0.67	36.6±3.6

As shown in Table 5.1, when the concentration of NH_3-N is higher than 18.69 mM, the microalgae growth in artificial wastewater would be limited by ammonia toxicity. This result is in accordance with the research conducted by Collos & Harrison (2014), which reported that threshold of ammonia toxicity to *Chlorophyceae* was about 23.76 mM. Compared with *Chlorophyceae*, the microalgae species of *Diatomophyceae*,

Raphidophyceae, *Dinophyceae*, and *Prymnesiophyceae* have much lower threshold of ammonia toxicity (Collos & Harrison, 2014). According to publications, some sources of wastewater, such as landfill leachate (Wett & Rauch, 2003), and digested sludge liquor (Campos et al., 2002), have high concentrations of NH_3-N. Therefore, ammonia toxicity is a potential problem to microalgae cultivation.

5.3.2. Hypothesis of NH_3-N assimilation

GS-GOGAT pathway has been proven to be the main metabolic pathway for NH_3-N assimilation in microalgae (Wu et al., 2016). Two enzymes, including glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT), are essential to GS-GOGAT pathway. In the GS-GOGAT pathway, glutamate is produced by the combination of NH_3-N and α-KG (Zuñiga et al., 2016). Glutamine synthesis and glutamate synthesis are shown in Eq. 6 and Eq. 7.

$$\text{Glutamate} + \text{ATP} + NH_3 \rightarrow \text{Glutamine} + \text{ADP} + \text{phosphate} \qquad \text{Eq. 6}$$

$$\text{Glutamine} + \alpha\text{-KG} + \text{NADH} \rightarrow \text{Glutamate} + \text{NAD} \qquad \text{Eq. 7}$$

In microalgae metabolism, α-KG is obtained from either intracellular environment or external environment (Guo et al., 2014). In microalgae metabolisms, α-KG is a product of Krebs cycle. When the intracellular source of α-KG is not enough, microalgae cells could absorb α-KG from external environment through cell membrane transport. Therefore, adding α-KG in the artificial medium should be a possible way to alleviate ammonia toxicity (Huo et al., 2011).

In microalgae cells, some of the absorbed organic carbon could be exploited for GS-GOGAT pathway while some of the organic carbon is utilized to synthesize fatty acids and polysaccharide (Koller et al., 2012). α-KG could be directly used by microalgae cells for NH_3-N assimilation. Hence, it should be a possible way to mitigate ammonia toxicity by adding α-KG into artificial wastewater.

5.3.3. α-KG assisted NH_3-N assimilation

As shown in Figure 5.1(a), the addition of α-KG promoted NH_3-N assimilation and algae growth. For example, NH_3-N removal efficiency increased from 6.32% to 36.73% with addition of α-KG. In addition, the addition of α-KG promoted the microalgae growth (Figure 6.2(b)).

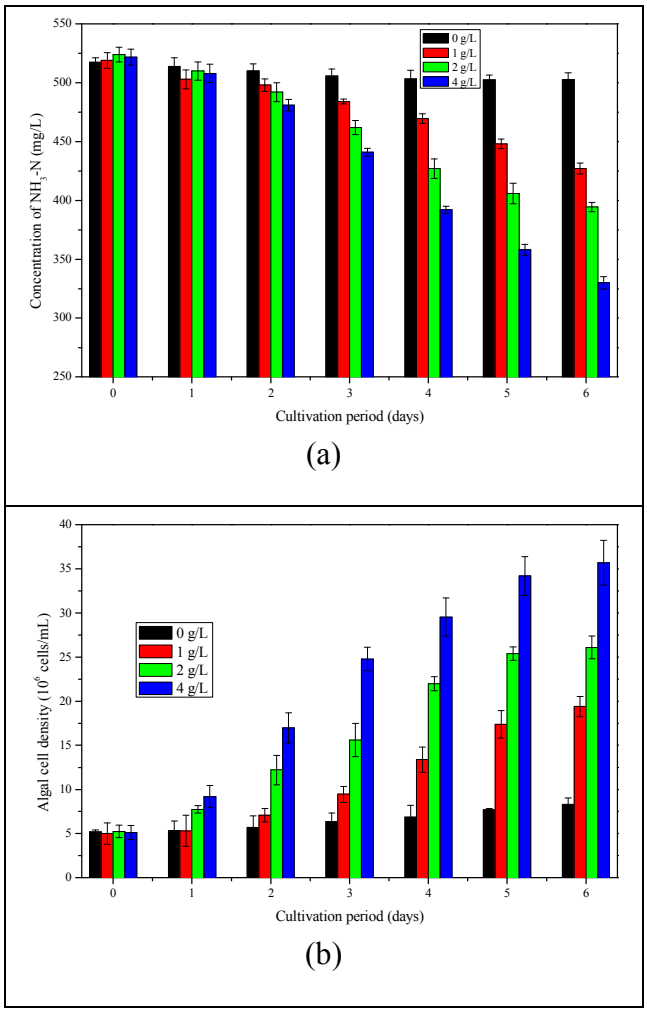


Figure 5.1. Addition of α-KG in artificial wastewater for algae growth and ammonia removal

The data in Table 5.2 showed that when the concentration of α-KG was lower than 2 g/L, experimental value of NH_3-N assimilation was much higher than theoretical value of NH_3-N assimilation. However, when the concentration of α-KG was higher than 2 g/L, the experimental value of NH_3-N assimilation was much lower than the theoretical value of NH_3-N assimilation. Because of the excessive α-KG in artificial wastewater, some of the absorbed α-KG was not utilized for the NH_3-N assimilation. Therefore, the low utilization efficiency of α-KG is a problem for ammonia assimilation in artificial wastewater with high concentration (>2 g/L) of α-KG.

Table 5.2. NH_3-N assimilation in wastewater with α-KG

Concentration of α-KG (g/L)	Concentration of α-KG (mM)	Theoretical NH_3-N removal (mg/L)	Experimental NH_3-N removal (mg/L)
1	4.42	61.9	91.9
2	8.84	123.7	119.6
4	17.70	247.8	171.8

Some of the α-KG that could not be utilized to assimilate NH_3-N, should be converted to oxaloacetate through Krebs cycle (Arous et al., 2016). Furthermore, because α-KG is not a cheap or common carbon source, in the practice, it is not a very good way to utilize the α-KG for microalgae cultivation and wastewater treatment. Based on the discussion above, it is necessary to find out common and cheap carbon sources to mitigate ammonia toxicity in wastewater.

5.3.4. Effects of common carbon sources on NH_3-N assimilation

Figure 5.2 showed that different carbon sources had very different impacts on NH_3-N assimilation in microalgae cells. For example, as shown in Figure 5.2(a), in artificial wastewater with bicarbonate NH_3-N removal efficiency was about 6.03%. Figure 5.2(b) and Figure 5.2(c) showed that removal efficiencies of NH_3-N in artificial wastewater with citric acid and glucose were much higher. Therefore, organic carbon is much more favorable to the ammonia assimilation (Alaba et al., 2017). Bicarbonate and carbon

dioxide could be utilized by microalgae cells by photosynthesis (Bond et al., 2001). In Calvin cycle, inorganic carbon could be converted to organic carbon. Compared with organic carbon utilization, inorganic carbon absorption and assimilation driven by photosynthesis are more time-consuming (Cuellar-Bermudez et al., 2015). Due to the low photosynthetic rate, algae may not accumulate enough glucose in a short time period for α-KG synthesis and NH_3-N removal. This is the main reason for the low removal efficiency of NH_3-N in artificial wastewater added with bicarbonate during 6 days cultivation. Although extension of cultivation period is a possible way to increase NH_3-N removal efficiency, long cultivation period will increase wastewater treatment cost and limit the application of algae technology (Manninen et al., 2016). Hence, addition of organic carbon, rather than inorganic carbon, is preferred in algae cultivation for NH_3-N removal.

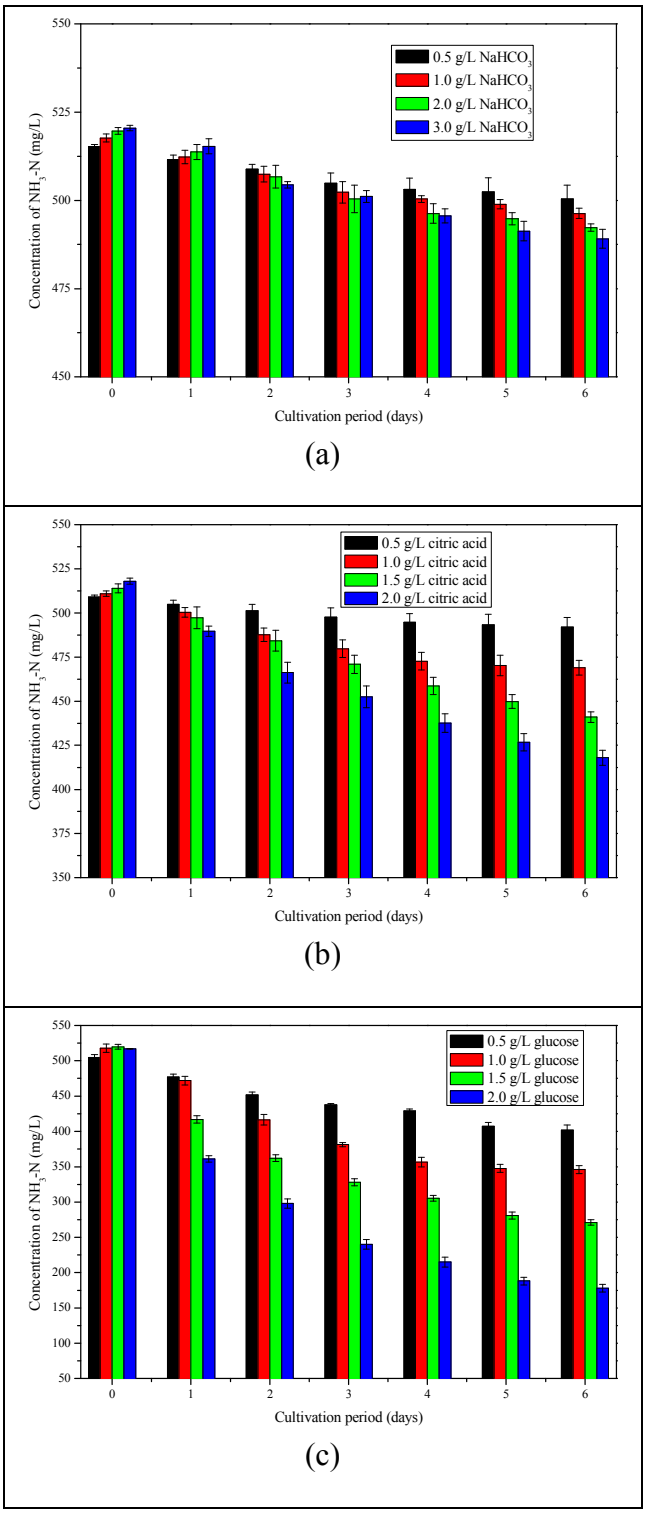


Figure 5.2. Effects of carbon sources on NH_3-N removal

Comparison between Figure 5.2(b) and Figure 5.2(c) suggested that microalgae cultivated in the artificial wastewater with glucose had much higher NH_3-N removal efficiencies.

Glucose could be converted to α-KG by glycolysis and Krebs cycle while citric acid is converted to α-KG by Krebs cycle. Therefore, glucose and citric acid have totally different abilities of producing ATP and NADH. For example, in the process of converting 1 mole glucose to α-KG, about 2 moles ATP and 6 moles NADH could be produced. But only 1 mole NADH could be produced when 1 mole citric acid is converted to 1 mole α-KG. Because of the different abilities of producing ATP and NADH, glucose and citric acid will have different performance in the removal of NH_3-N (Theodosiou et al., 2017). In this chapter, the barrier to NH_3-N assimilation is the deficiency of energy and hydride donor.

In this study, because of the photosynthesis, organic carbon in the artificial wastewater was not the only carbon source for microalgae growth (Kumar et al., 2014). In order to eliminate the effects of photosynthesis on carbon utilization and NH_3-N assimilation, light source was removed in the following experiment.

5.3.5. Termination of photosynthesis for NH_3-N assimilation

In Figure 5.3, removal efficiencies of NH_3-N in artificial wastewater added with glucose could reach 44.31% while the removal efficiencies NH_3-N in artificial wastewater with citric acid were much lower than 7.14%. In this study, organic carbon in wastewater was the only carbon source for microalgae growth.

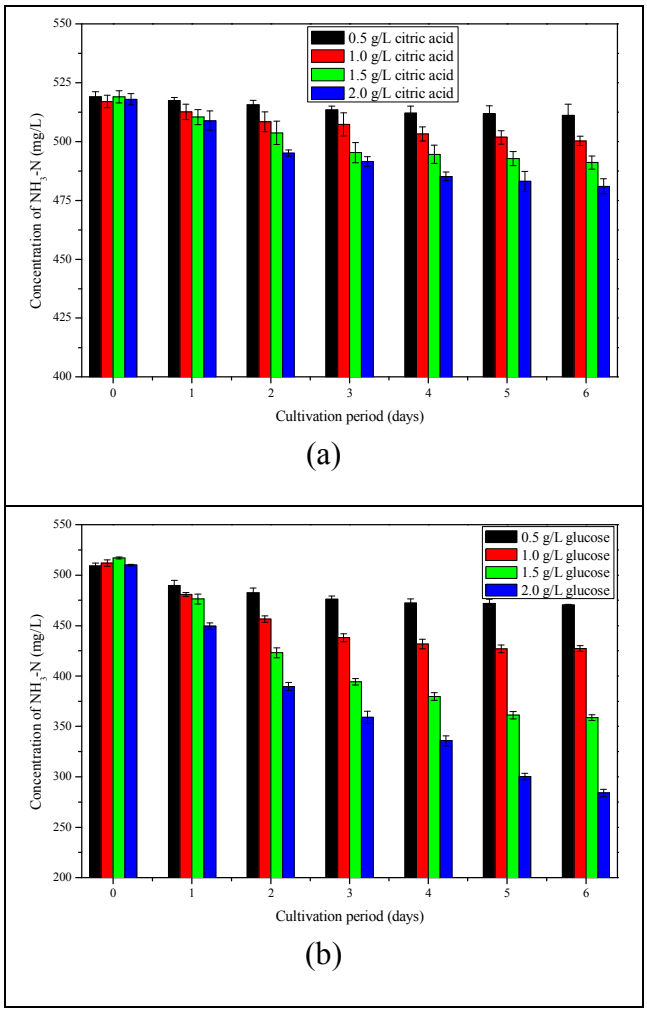


Figure 5.3. Effects of two organic carbon sources on NH_3-N removal in dark

As shown in Table 5.3, experimental value of NH_3-N assimilation was much lower than theoretical value of NH_3-N assimilation in artificial wastewater with organic carbon. The most possible reason is that microalgae only utilized some of organic carbon for ammonia assimilation. Figure 5.3(a) and Figure 5.3(b) indicated that in artificial wastewater added with glucose, more absorbed carbon was utilized by microalgae for ammonia assimilation. Compared with citric acid, glucose could produce much more energy and hydride donor. With sufficient energy and hydride donor for microalgae growth, more α-KG could be utilized for NH_3-N assimilation.

Table 5.3. NH_3-N removal in wastewater with organic carbon source in dark

Carbon source	Content (g/L)	Utilization efficiency (%)	Theoretical NH_3-N removal (mg/L)	Experimental NH_3-N removal (mg/L)
Glucose	0.5	96.0%	37.3	29.3
	1.0	92.0 %	71.5	65.4
	1.5	90.6%	105.8	127.5
	2.0	91.5%	142.3	167.4
Citric acid	0.5	90.2%	32.8	7.2
	1.0	86.0%	61.3	15.1
	1.5	85.3%	80.9	25.4
	2.0	79.5%	97.7	34.5

According to the results of this study, it is confirmed that glucose with the ability of producing more energy and hydride donor is better than citric acid for the assimilation of NH_3-N.

5.3.6. Discussion of strategies to alleviate ammonia toxicity

5.3.6.1. Carbon source for NH_3-N assimilation

The study of Lu et al. (2016) indicated that microalgae grown in dairy processing wastewater removed around 200 mg/L NH_3-N in two days. However, the contribution of carbon sources to the NH_3-N removal has not been reported. This chapter focused on three common carbon sources, glucose, citric acid, and bicarbonate. The result indicated

that organic carbon with the ability of producing more energy and hydride donor could be more favorable to NH_3-N assimilation in microalgae cells.

5.3.6.2. Comparison of ammonia toxicity alleviation strategies

In previous publication, concentration of ammonia in wastewater is reduced by ammonia stripping (Han et al., 2013). It was reported that ammonia stripping could remove 92.8% NH_3-N from wastewater (Guštin & Marinšek-Logar, 2011). However, the emission of ammonia gas into air atmosphere could cause potential environmental pollution. In addition, the ammonia in wastewater could be converted into nitrate by nitrifying bacteria. Liu et al. (2016) reported that more than 95% NH_3-N in the wastewater could be converted to NO_3-N. However, it took about 60 days to enrich nitrifying bacteria in the wastewater (Liu et al., 2016). The long treatment period seriously limits the use of this strategy in the wastewater-based microalgae cultivation.

5.3.6.3. Practical application

In the practice, it is not a promising way to add glucose in wastewater to for microalgae cultivation and NH_3-N assimilation since glucose is not a very cheap carbon source. According to the publications, some food processing wastewater, such as molasses waste stream (Tsioptsias et al., 2015), dairy processing wastewater (Martín-Rilo et al., 2015), and fruit processing wastewater (Rahim & Raman, 2015), have very high concentrations of saccharides. These sources of wastewater could be used to alleviate ammonia toxicity and promote microalgae growth at low cost (Sinsabaugh et al., 2014).

5.4. Conclusions

The conclusions of this chapter include: (1) Ammonia toxicity became a problem to microalgae cultivation when the concentration of NH_3-N was higher than 28.03 mM; (2) It is not practical to use α-KG to mitigate ammonia toxicity in microalgae cultivation because of its high cost; (3) Compared with inorganic carbon and citric acid, glucose has much better performance in ammonia removal; (4) It is hypothesized that organic carbon with ability of producing more energy and hydride donor is better for the NH_3-N assimilation.

Chapter 6. Cooperation between algae and wastewater-borne bacteria in nutrients metabolism

6.1. Introduction

Centrate wastewater, generated from the centrifugation of activated sludge in municipal wastewater treatment plant, has various nutrients, which may be utilized by microalgae (Li et al., 2011). Previous study indicated that the cultivation of *Chlorella* sp. could remove about 61% total nitrogen (TN), 70% chemical oxygen demand (COD), and 61% total phosphorus (TP) in the centrate wastewater (Min et al., 2011). Microalgae cultivated in centrate wastewater with sufficient nutrients have very high biomass yield (Pittman et al., 2011).

Wastewater-borne bacteria, which could compete with microalgae for nutrients, seriously threaten the microalgae growth in wastewater (Fergola et al., 2007). Some strategies, including the sterilization at high temperature and the use of antibiotics, have been widely applied to limit bacteria in wastewater-based microalgae cultivation (Jemli et al., 2002; Lu et al., 2015). Some publications showed that microalgae and some bacteria could have cooperation in the wastewater treatment. For example, the co-immobilization of *Chlorella sorokiniana* and *Azospirillum brasilense* was able to remove more nutrients in municipal wastewater (De-Bashan et al., 2004). In addition, Croft et al. (2005) increased biomass yield by growing *Chlorella vulgaris* with some bacteria. In recent years, more and more studies are focusing on the cooperation between microalgae and bacteria.

The studies on individual bacterial strain and microalgae could identify the cooperation relationship in the algal-bacterial system (Buchan et al., 2014). Furthermore, the isolated bacteria could be added into wastewater to promote microalgae cultivation (De‐Bashan

et al., 2008). There are various cooperation model in the algal-bacterial system. First, microalgae could utilize the nutrients released by bacteria (Lu et al., 2013). For example, the bacteria in wastewater could convert solid particles into soluble nutrients by producing extracellular enzymes, such as amylase, lipase, and protease (Buchan et al., 2014; Pohlon et al., 2010). Second, some bacteria could produce vitamins which are favorable to the growth of microalgae (Croft et al., 2005). Third, carbon dioxide produced by aerobic bacteria could be favorable to the growth of microalgae (Boschker et al., 2005).

It was reported that some wastewater-borne bacteria could promote the growth of microalgae. However, the cooperation model between algae and bacteria in has not been fully understood yet (Ma et al., 2014). Some challenging questions need to be solved include: (1) How could microalgae and wastewater-borne bacteria cooperate with each other in the wastewater? (2) What is the function of wastewater-borne bacteria in the algal-bacterial system? and (3) What are the characteristics of bacteria that are favorable to the growth of microalgae?

This chapter has four experimental steps: First, microalgae were cultivated in municipal wastewater with bacteria in a pilot-scale system. Second, nutrients removal and biomass yield in the batch experiment were measured. Third, a bacterial strain that was favorable to the microalgae growth was isolated and identified. Final, the cooperation between the isolated wastewater-borne bacteria and microalgae was studied.

6.2. Materials and methods

6.2.1. Centrate wastewater

Basic characteristics of the wastewater used for microalgae cultivation are shown in Table 6.1. The neutral pH value is favorable to the microalgae growth. In addition, in this wastewater, there were many nutrients, such as nitrogen, phosphorus and some other organics. These nutrients are important to the microalgae growth. Thirdly, because of the positive value of ORP, the centrate wastewater was in an aerobic condition.

Table 6.1. Basic characteristics of centrate wastewater

Parameter	Values	Parameter	Values
TN	42.87±5.62 mg/L	NH_3-N	31.72±0.38 mg/L
TP	36.91±3.77 mg/L	COD	836.67±11.24 mg/L
ORP	111.00±8.66 mV	pH	6.26±0.46
CFU	$0.96\times10^6\pm0.04\times10^6$	TVSS	1.12±0.18 g/L

6.2.2. Parameters measurement

In this chapter, two parameters, including ORP and pH value, of the wastewater measured daily. Some essential nutrients, such as TN, TP, COD, and NH_3-N, of the wastewater were analyzed daily. The measurement was conducted by using a spectrophotometer according to the method in publication (Lu et al., 2016). Concentrations of nutrients were expressed as mg/L.

Algae and wastewater-borne bacteria were separated by a filter membrane. The biomass yield of algae and bacteria was calculated based on the measurement of TVSS (Lu et al., 2015). Average growth rate (g/L/d) of algae was calculated according to Eq. 1.

$$R = (T_a - T_0)/t \quad \text{Eq. 1}$$

where R is the average growth rate of algae; T_a and T_0 are the TVSS of algae at Day *a* and Day *0*, respectively; t is the time interval (days).

6.2.3. Pilot-scale bioreactor

The pilot-scale bioreactor used for the treatment of wastewater and microalgae cultivation had two retention tanks and a three-layer photo-bioreactor (PBR). The volumes of retention tanks and PRB were about 400L and 900L, respectively. In the experiment, temperature and relative air humidity were controlled around 28±5 ^{o}C and 50±10%.

6.2.4. Batch experiment

In the batch experiment, *Chlorella* sp. was preserved in artificial wastewater (Hollinshead et al., 2014). Before inoculation into wastewater, microalgae cells were washed for two times by using stilled water.

Batch experiment was conducted in lab environment. Algae were cultivated in 250 mL flasks with 100 mL medium or wastewater. The temperature and relative air humidity in the batch experiment were controlled at 25±1 ^{o}C, and 45±3%.

6.2.5. Isolation and identification of bacteria

At the end of batch experiment, a bacterial strain was isolated from the wastewater according to the method reported by Liu et al. (2016). The isolated bacteria were preserved on solid beef extract medium. The nutrient profile of this medium includes: NaCl (15.0 g/L), peptone (10.0 g/L), beef extract (5.0 g/L), and agar (20.0 g/L).

Total DNA of the isolated bacteria was extracted by the DNA Extraction Kit (MP Biomedicals, USA) according to manufacturer's instructions. The PCR program used in this work has been reported by previous publication (Liu et al., 2016). The profile of bacteria in wastewater during the batch experiment was measured by high-throughput sequence analysis. This step was supported by the Biology Department of University of Minnesota (Liu et al., 2016). The isolated bacterial strain and bacterial strains reported in previous publications were compared (Buchanan & Gibbons, 1974).

6.2.6. Co-cultivation of algae and isolated bacterial strain

In this work, the wastewater was autoclaved for 30 min at 121 ^{o}C before microalgae cultivation. The bacterial strain isolated from wastewater were inoculated in the sterilized wastewater. The microalgae cultivation conditions, such as temperature and relative air humidity, strictly controlled.

6.3. Results

6.3.1. Wastewater treatment at a pilot-plant scale

As shown in Table 6.2, wastewater had very different initial concentrations of nutrients. The main reason for this is that the wastewater was received from the wastewater treatment plant at different time. With the growth of microalgae, average concentrations of nutrients, including TN, TP, NH_3-N and COD, in the wastewater decreased to 65.7 mg/L, 15.0 mg/L, 41.1 mg/L, and 430 mg/L, respectively.

Table 6.2. Concentrations of nutrients in wastewater during pilot-scale treatment

		1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
TN	Initial	131.0	203.5	124.9	89.1	102.2	109.0	86.2
	Final	80.6	123.6	72.8	50.4	46.3	50.2	35.8
TP	Initial	57.4	112.4	59.6	39.8	101.4	120.8	66.3
	Final	16.2	36.4	12.8	5.8	11.0	11.1	11.5
NH_3-N	Initial	99.8	120.6	67.5	61.1	65.4	68.7	57.6
	Final	55.6	68.9	40.5	35.4	29.2	33.7	24.7
COD	Initial	1704	1832	1027	1272	2113	2184	1801
	Final	753	795	300	270	351	287	256

As shown in Figure 6.1, nutrients removal increased week by week (Figure 6.1(c)). For example, removal efficiencies of these nutrients, COD, TP, TN, and NH_3-N, in the Week 7 were 29.98%, 10.83%, 20.00%, and 12.92%, higher than the removal efficiencies in the Week 1. The main reason for this phenomenon is that the cooperation between

microalgae and wastewater-borne bacteria in the wastewater increased the nutrients removal efficiencies gradually (de-Bashan & Bashan, 2010). In the last three weeks of this experiment, the cooperation between microalgae and bacteria became stable.

As shown in Figure 6.1, bacteria and microalgae grew at the same time in this pilot-scale system. Figure 6.1(a) indicated that the biomass yield of microalgae increased by 0.28~0.39 g/L in this experiment. As shown in Figure 6.1(b), CFU of bacteria in the wastewater increased by more than 15 times. The CFU of bacteria in wastewater algae one-week treatment was higher than 1.67×10^7. Therefore, in this wastewater, microalgae and bacteria could survive together. The ORP value of the wastewater was higher than 0 mV. Therefore, under the aerobic conditions in this wastewater, aerobic bacteria should be the dominant species.

Figure 6.1(d) indicated that the ORP value of the wastewater decreased gradually during microalgae cultivation. This result suggested that this wastewater treatment process consumed oxygen gas but the wastewater was still under aerobic conditions. Because of the photosynthesis, the activity of microalgae cells would produce oxygen gas. However, the activity of bacteria would reduce the ORP value in the wastewater. In order to study the metabolic mechanisms of algal-bacterial community in this wastewater, batch experiment was conducted.

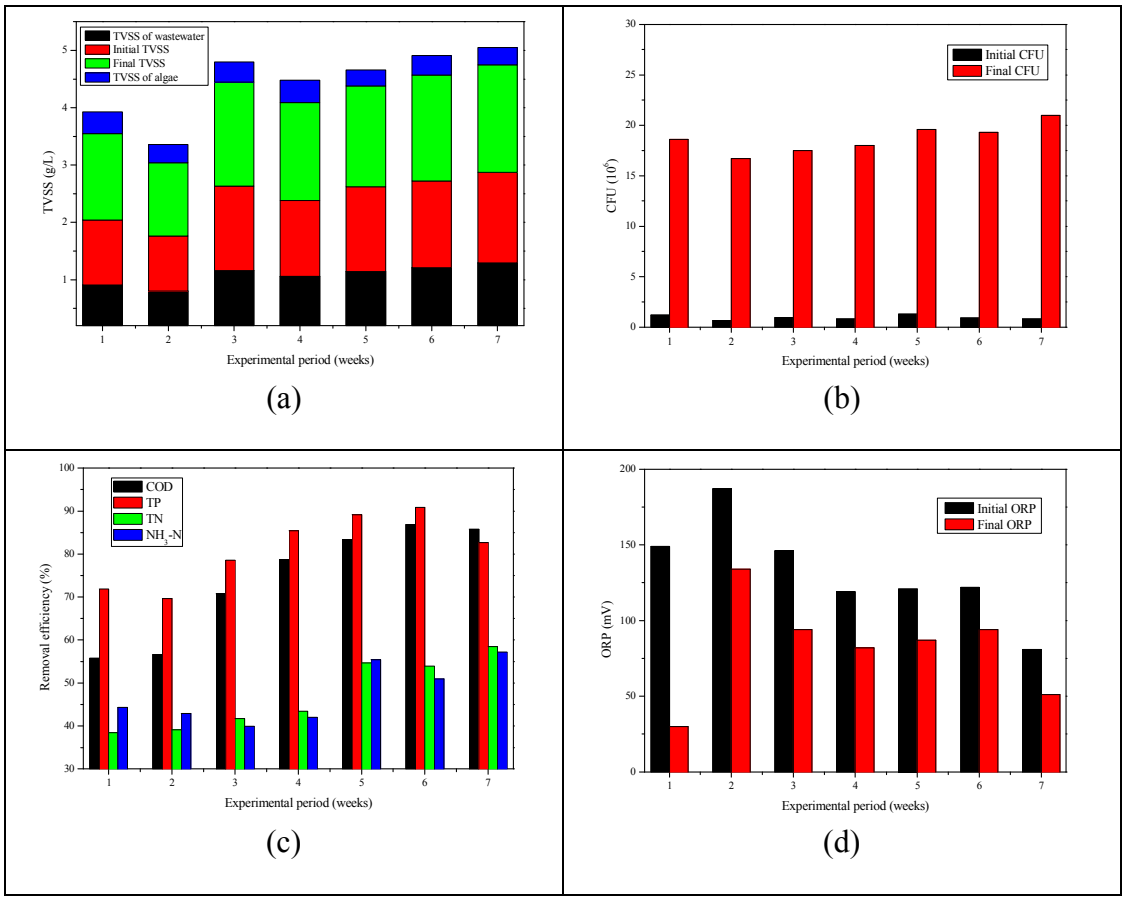


Figure 6.1. Algae growth and nutrients removal in pilot scale system

6.3.2. Batch experiment

As shown in Figure 6.2(a), biomass yield in the wastewater increased gradually. Figure 6.2(d) suggested that the microalgae biomass mainly contributed to the increase of biomass yield. According to the ratio of microalgae to bacteria, microalgae biomass is the dominant biomass in algal-bacterial system.

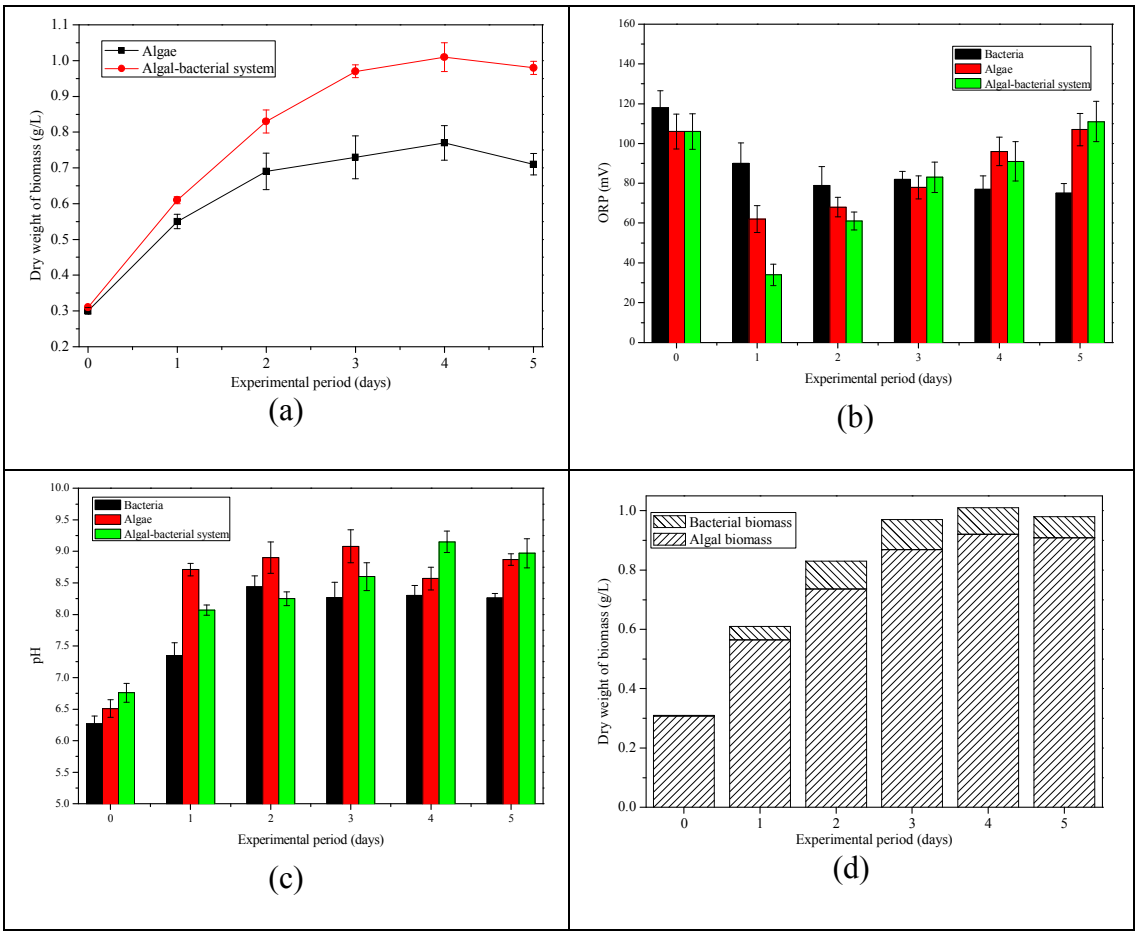
Figure 6.2(b) indicated that the growth of bacteria in wastewater is an oxygen-consuming process while the growth of microalgae is an oxygen-producing process. Because of the positive values of ORP, the wastewater was in an aerobic condition. In such an

environment, the aerobic bacteria should be dominant in the bacterial community (Chen et al., 2012). These results agree with the experimental results in the pilot-scale system. In this study, two possible reasons are proposed to explain the changes of ORP values of the wastewater. First, the photosynthesis rate of newly inoculated microalgae was not high enough (Li et al., 2011a). As a result, the activities of bacteria in the wastewater reduced the ORP values. However, with the growth of microalgae, more oxygen gas was produced and the ORP value increased gradually. Second, because of the organic carbon in wastewater, microalgae were in a heterotrophic growth model. As a result, a lot of oxygen was consumed at the beginning. This explanation is partly supported by the changes of the concentrations of COD in the wastewater (Figure 6.2(f)).

As shown in Figure 6.2(c), the metabolisms of microalgae and bacteria in the wastewater contributed to the increase of pH value. Vlek & Stumpe (1978) reported that ammonia volatilization mainly happened when the pH value of aqueous phase was higher than 9.5 (Vlek & Stumpe, 1978). In this research, pH value of wastewater was lower than 9.5. Therefore, the external environment could not significantly cause the removal of ammonia. It should be the metabolisms of microalgae and bacteria that caused the nutrients removal in this wastewater.

As shown in Figure 6.2(e), bacteria only removed about 16.84% NH_3-N in the wastewater without microalgae. When microalgae and bacteria grow together in the wastewater, removal efficiency of NH_3-N was around 90.66%. The co-cultivation of microalgae and bacteria also contributed to the high removal efficiency of TN (Figure 6.2(g)). According to the data in Figure 6.2(g), wastewater-borne bacteria only removed 13.64% TN while microalgae removed 77.14% TN. Therefore, the metabolisms of

microalgae mad more contribution to TN removal. Figure 6.2(f) indicated that the removal efficiencies of COD in wastewater with bacteria microalgae reached 72.56% and 74.57%, respectively. So in the wastewater there are a lot of indigestible nutrients that could not be utilized by microalgae or bacteria. However, the algal-bacterial community removed about 93.01% COD in the wastewater. Figure 6.2(h) indicated that bacteria and microalgae only removed 52.65% and 86.81% TP, respectively, while the co-cultivation of microalgae and bacteria removed about 98.78% TP. According to this result, in terms of COD removal and TP removal, co-cultivation of microalgae and bacteria is much better than individual bacterial or algal community.



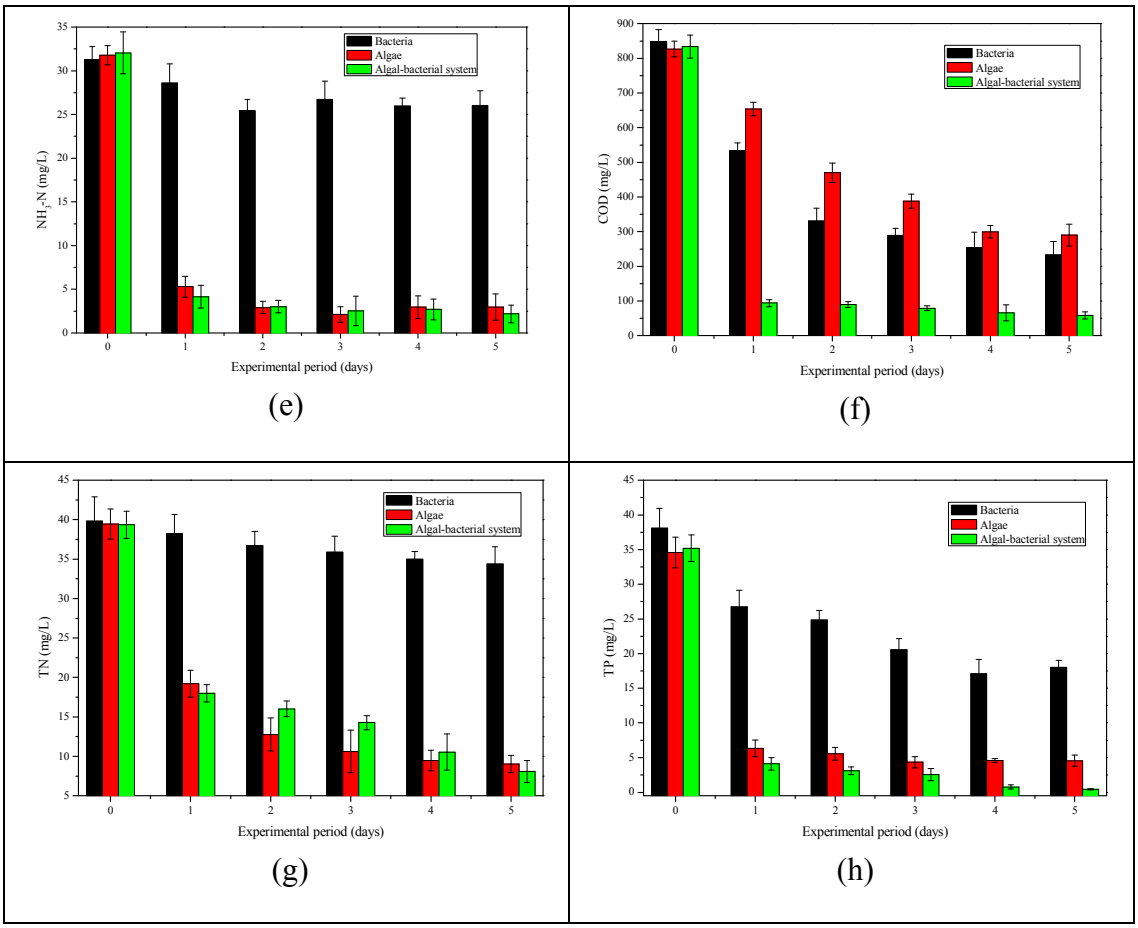


Figure 6.2. Algae growth and nutrients removal in batch experiment

Two possible reasons are responsible for the better performance of algal-bacterial system in nutrients removal. First, in the wastewater, aerobic bacteria, which was the dominant bacterial community, and microalgae exchange carbon dioxide and oxygen (Kumar et al., 2010; Su et al., 2011). Such a cooperation model promoted the growth of microalgae and bacteria. Second, the metabolisms of bacteria convert some indigestible nutrients to soluble nutrients (More et al., 2014). To find out the cooperation model between microalgae and bacteria, specific bacterium was isolated from the wastewater and identified accordingly.

6.3.3. Changes of bacterial community in wastewater treatment

Figure 6.3 showed that the bacterial community changes a lot in the cultivation period. For example, the percentage of *Acinetobacter* sp. in wastewater before microalgae cultivation was about 13.20% but increased to 60.43% after microalgae growth. Therefore, *Acinetobacter* sp. became the dominant bacterial strain in wastewater. At the end of cultivation, the bacterial strains with high abundance (above 5%) were aerobic bacteria, including *Acinetobacter* sp. (60.43%), *Brevundimonas* sp. (7.35%), and *Rhodocyclaceae* sp. (7.99%). The main reason for the high abundance of aerobic bacteria is that the wastewater was remained in aerobic conditions. Two factors, including oxygen dissolving and metabolisms of algae, mainly contributed to the aerobic conditions of the wastewater. Firstly, in batch experiment, the flasks were shaken continuously. Accordingly, the oxygen in air was dissolved in the wastewater. Secondly, through photosynthesis, algae generated oxygen gas which also contributed to the formation of aerobic environment in the wastewater. So the synergistic relationship was established on the cooperation between algae and aerobic bacteria.

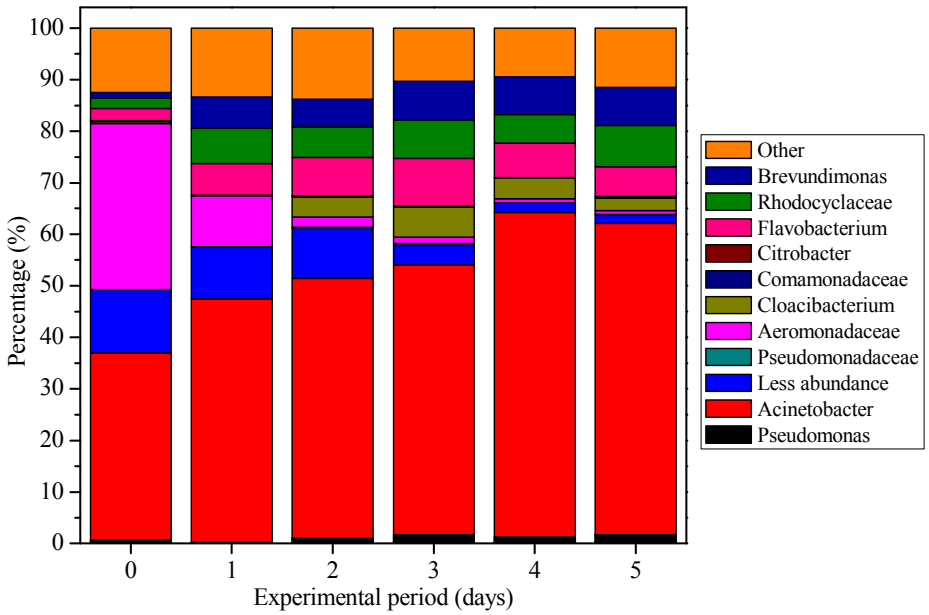


Figure 6.3. Changes of bacterial community in wastewater treatment

6.3.4. Isolation and identification of bacterial strain

6.3.4.1. Genetic identification of isolated strain

The results in Table 6.3 indicated that the proximities between this isolated bacterium and some reported bacteria, including *Acinetobacter towneri*, *Acinetobacter genomic*, and *Acinetobacter haemolyticus*, were about 96.87%, 96.69%, and 95.13%, respectively. Therefore, the isolated bacterial strain was identified as *Acinetobacter* sp.

Table 6.3. Homology between 16s rRNA gene sequences of isolated strain and GenBank strains

rRNA length (bp)	Relevant bacterial strains	Proximity
1417	*Acinetobacter towneri* (AB 1110)	96.87%
	Acinetobacter genomic (ATCC 17979)	96.69%
	Acinetobacter haemolyticus (ATCC 17906)	95.13%
	Acinetobacter calcoaceticus (DSM 30006)	95.31%
	Acinetobacter baumannii (ATCC 19606)	96.65%
	Acinetobacter lwoffii (ATCC 17925)	95.23%
	Acinetobacter baylyi (CCM 7195)	96.84%
	Acinetobacter junii (LMG 998)	96.01%

7.3.4.2. Morphological and biochemical characteristics

The results of Table 6.4 indicated that this isolated bacterium is gram-negative bacilli without oxidase. First, because of the negative glucose fermentation test, this isolated bacterium could not utilize the glucose in wastewater. The positive DL-lactate utilization test and citrate utilization test suggested that this isolated bacterium mainly utilized non-glucose carbon source. Second, because of the positive nitrate reduction test and nitrite reduction test, this isolated bacterium should have the ability of denitrification. Third, the positive oxygen demand test suggests that the isolated bacterium is an aerobic bacterium.

Table 6.4. Morphological and biochemical characteristics of isolated strain

Characteristics	Results	Characteristics	Results
Shape	Rod	Gram reaction	-
Size	(1.0-1.5)μm × (1.5-2.5)μm	Oxidase	-
Glucose fermentation	-	Indole production	-
Contact enzyme	+	Nitrate reduction	+
Nitrite reduction	+	Oxygen demand	+
DL-lactate utilization	+	Citrate utilization	+

According to the genetic identification and morphological and biochemical analysis, the bacterium isolated from the wastewater is *Acinetobacter* sp. In the publications, *Acinetobacter* sp. is a bacterium widely surviving in wastewater, activated sludge, and soil. It was reported that this bacterium is a type of non-fermentative aerobic microorganism (Gao et al., 2014; Wang et al., 2013a; Weon et al., 2002). Some publications tried to develop some strategies to enrich *Acinetobacter* sp. in wastewater to remove phosphorus (Yang et al., 2015; Zhou et al., 2010). In some studies, *Acinetobacter* sp. was also used to degrade some non-glucose carbon, such as oleic acid, acetate, phenol, and ethanol, in the wastewater (Bouvet & Grimont, 1986; Li et al., 2001; Ying et al., 2007). To my knowledge, the co-cultivation of microalgae and *Acinetobacter* sp. in wastewater to remove nutrients and produce biomass has not been studied by the publications.

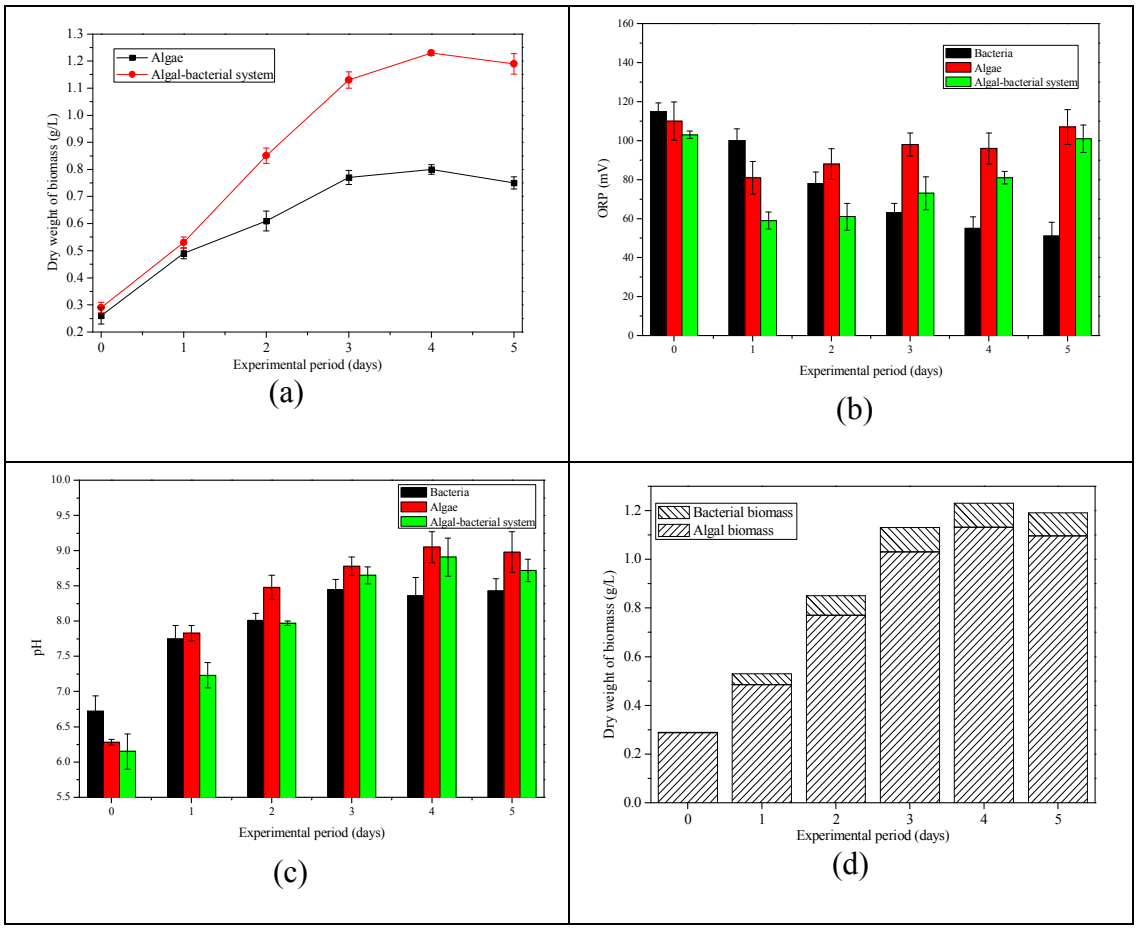
6.3.5. Co-cultivation of algae and *Acinetobacter* sp. in wastewater

In the batch experiment, there were various bacterial species in the algal-bacterial system. Therefore, it is not possible to find out the exact cooperation model between microalgae and bacteria. In this section, the cooperation model between microalgae and the isolated bacterium, *Acinetobacter* sp. was studied. Since the isolated bacterium was the dominant bacterium in the bacterial community, this study could reflect the cooperation between bacteria and microalgae.

As shown in Figure 6.4(d), the ratio of bacterial biomass to algal biomass was not high in the wastewater treatment. In this way, microalgae have more advantages in the competition with bacteria for nutrients. This result agrees with the previous publications. According to data in Figure 6.4(d), the increase of microalgae biomass mainly contributed to the increase of biomass. This result suggested that the existence of *Acinetobacter* sp. has a positive effect on the growth of microalgae.

Figure 6.4(b) showed that ORP value of the wastewater was positive, suggesting that the wastewater was in an aerobic condition. Also because of the low pH value of wastewater, ammonia volatilization and phosphorus sedimentation were mainly attributed by the microorganism activities (Figure 6.4(c)). Figure 6.4(e) indicated that in this wastewater, *Acinetobacter* sp. did not have great ability of utilizing NH_3-N while it is the activity of microalgae that mainly removed NH_3-N. Figure 6.4(g) indicated that *Acinetobacter* sp. and microalgae only removed 13.54% and 73.10% of TN while the removal efficiency of TN reached 79.12% of TN in wastewater with both bacteria and microalgae. This result showed that the co-cultivation of microalgae and bacteria was favorable to the removal of

TN in wastewater. Figure 6.4(h) indicated that the the TP removal efficiency increased to 96.26% in the wastewater with algal-bacterial community. Similar phenomenon was observed in the removal of COD (Figure 6.4(f)). Therefore, the cooperation between microalgae and *Acinetobacter* sp. was important to the removal of nutrient in centrate wastewater. At the end of wastewater treatment, concentrations of residual nutrients, including COD, TN, and TP, were 178 mg/L, 8.99 mg/L, and 0.97 mg/L, respectively. According to the discharge standards of municipal wastewater, after treatment, this wastewater reached the permissible discharge limit.



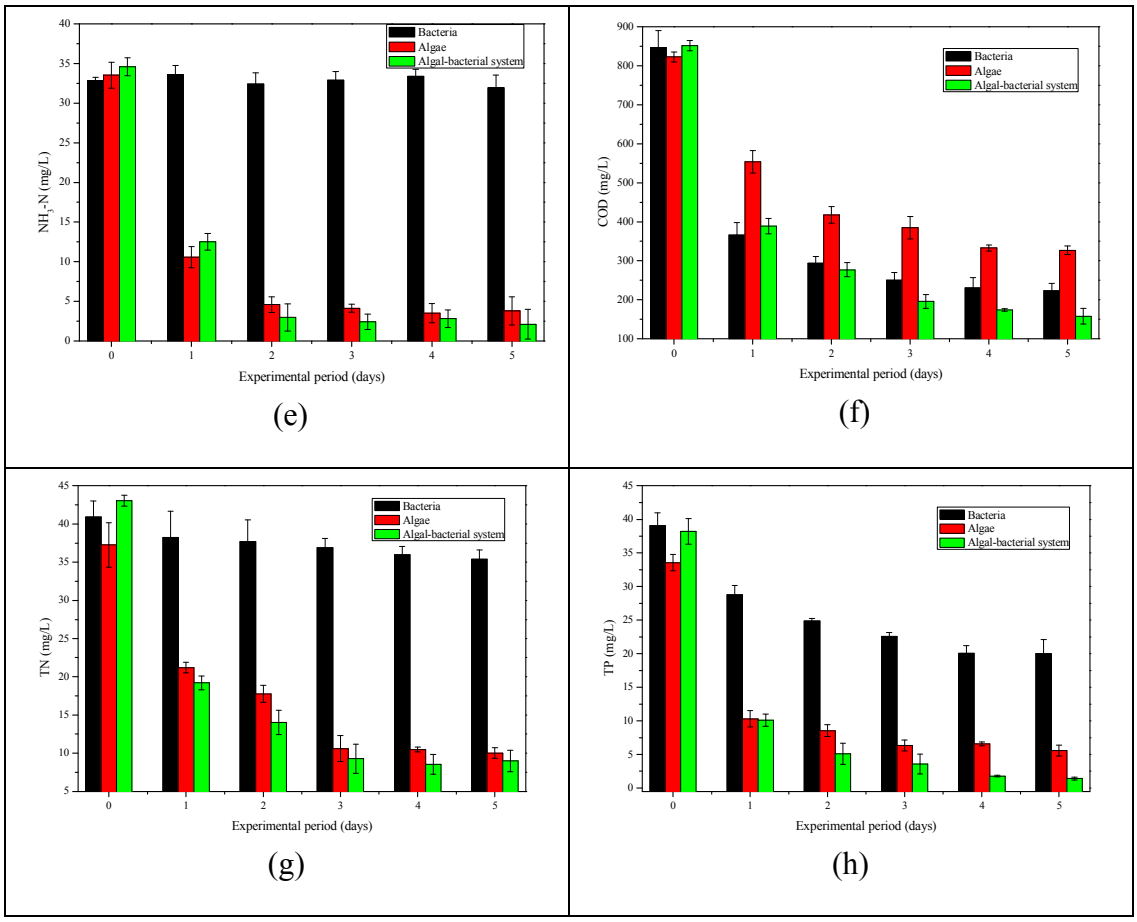


Figure 6.4. Biomass yield of algae, changes of ORP and pH, and removal efficiencies of nutrients in the combined system of algae and *Acinetobacter* sp.

6.3.6. Cooperation between algae and *Acinetobacter* sp.

Experimental results showed that the activities of *Acinetobacter* sp. promoted the microalgae growth and nutrients removal in wastewater. The potential cooperation between microalgae and *Acinetobacter* sp. is discussed in detail in this section.

First, in the wastewater treatment, *Acinetobacter* sp. was important to the removal of phosphorus since *Acinetobacter* sp. has the metabolic pathways to utilize phosphorus. This point has been reported by some previous publications in the study of *Acinetobacter*

sp. for phosphorus removal (Akpor & Muchie, 2010; Wang et al., 2008). Second, *Acinetobacter* sp. could promote the growth of microalgae. As a result, the growth of microalgae promoted the phosphorus removal. Based on the discussion above, *Acinetobacter* sp. should have two potential ways to promote the phosphorus removal in the wastewater.

Second, it was supposed that the competition between microalgae and *Acinetobacter* sp. was not serious because there were sufficient nutrients at the beginning of wastewater treatment. As a result, the co-cultivation of microalgae and *Acinetobacter* sp. was established since there was no very intensive competition. In addition, in the co-cultivated microalgae and bacteria system, a wide range of organic carbon could be utilized (Mellado et al., 2013; Snellman et al., 2002). Therefore, the algal-bacterial system removed much more organic carbon in the centrate wastewater.

Third, microalgae started to absorb the inorganic carbon source by photosynthesis with the exhaustion of organic carbon. In this way, the microalgae cells transferred from heterotrophic model to phototrophic model (Deng et al., 2012; Wang et al., 2011). Since a lot of biomass was accumulated in the initial state of wastewater treatment, microalgae have great ability of producing oxygen gas (Price et al., 2012). The oxygen gas produced by microalgae would support the growth of *Acinetobacter* sp. As a result, in the wastewater, *Acinetobacter* sp. utilized the oxygen gas produced by microalgae while microalgae utilized the CO_2 produced by *Acinetobacter* sp. This cooperation model promote the growth of algal-bacterial community and the nutrients removal in the wastewater (Vardon et al., 2011).

6.4. Conclusions

The conclusions of this chapter include: (1) A bacterium was isolated from the wastewater and identified as *Acinetobacter* sp.; (2) The algal-bacterial system in wasteater improved the removal efficiencies of some nutrients; (3) After wastewater treatment, according to the concentrations of residual nutrients and discharge standard, the wastewater reached permissible discharge limit; (4) Based on the experimental results, the cooperation model between microalgae and bacteria was attributed to the exchange of CO_2 and oxygen between microalgae and bacterial; (5) The isolated bacterium, *Acinetobacter* sp. could be co-cultivated with microalgae for the wastewater treatment.

Chapter 7. Turbidity reduction and ammonia stripping of digested swine manure

7.1. Introduction

Soil degradation and environmental pollution caused by the intensive use of artificial fertilizer have become serious problems prohibiting the sustainable development of human society (Liu et al., 2010). To solve these problems, recently, the use of algal bio-fertilizer, which could protect the soil fertility, increase the yield of crops, and reduce the pollution, has attracted people's attention (Wang et al., 2015). However, high cost of algae cultivation is a problem limiting the wide use of algal bio-fertilizer. To reduce the cost of algae biomass, researchers have tried to use waste effluents, such as slaughterhouse wastewater, agricultural effluent, and food processing wastewater for algae cultivation (Ferreira et al., 2017; Hernández et al., 2016). It has been regarded as a promising way to exploit waste effluent for algae cultivation and use algae to recycle the nutrients (Liu et al., 2016).

Swine manure, an eutrophic agricultural waste effluent, is a potential resource for algae cultivation (Deng et al., 2018). However, swine manure contains a lot of suspended solids, which could not be directly assimilated by algal cells (Wang et al., 2010). In previous studies, before algae inoculation, swine manure was subjected to anaerobic digestion, which converted some solids to soluble nutrients, particularly short-chain fatty acids (Hu et al., 2013; Mulbry et al., 2008). The study of Hu et al. (2012) demonstrated that appropriate anaerobic digestion increased the concentrations of acetic acid and propionic acid in swine manure by 50%. (Wang et al., 2010) found that anaerobic digestion reduced the content of solids in manure from 8.00% to 5.10%. Compared with original manure, digested manure yield more algae biomass (Hu et al., 2013). Although anaerobically

digested swine manure (AD-SM) has been proven to be a good effluent for algae cultivation by researchers, in the wastewater treatment plant, AD-SM is rarely treated by algae.

One barrier to the commercialization of algae-based AD-SM treatment is the high consumption of freshwater. The dilution with freshwater could reduce the turbidity of AD-SM and create a better environment for algae growth. In the research of Wang et al. (2010), dilution ratio was 20-fold, meaning 19 L freshwater should be used for the treatment of 1 L anaerobically digested manure. In some cases, the dilution ratio of AD-SM even reached 100-fold (De la Noüe & Basseres, 1989). From either economic perspective or environmental perspective, it is not possible to use highly diluted AD-SM for algae cultivation in practice. To reduce the consumption of freshwater, Deng at al. (2017) and Deng et al. (2018) conducted vacuum-assisted thermophilic anaerobic digestion and recycled some post-harvest culture broth by centrifugation. However, thermophilic digestion, vacuum treatment, and high speed centrifugation would significantly increase the energy input and the operation cost. Because of these disadvantages, this newly developed technology is not feasible in the wastewater treatment plant (Deng et al., 2017). Therefore, it is essential to develop a cheap and simple pathway to pretreat AD-SM for algae cultivation in pilot scale system.

To remove some residual solids in waste effluent after anaerobic digestion, previous studies have used various affordable flocculants, such as aluminum sulfate, poly aluminum chloride, and polyacrylamide (de Paula et al., 2014; Žarković et al., 2011). The flocculating functions were expressed in two main ways, combining suspended particles by functional groups or reducing the repulsive force between particles by neutralizing

their surface electric charge (Barakat, 2011; Zhang et al., 2010). However, due to the toxicity of these flocculants or their degradation products, the use of algae grown in the waste effluent after flocculation would be limited. Starch is a cheap and non-toxic flocculating agent that widely applied in wastewater pretreatment. Hydroxyl functional group on starch could promote the attachment between suspended solids and further cause sedimentation (Wang et al., 2013b). However, starch could not accelerate flocculation by changing the electric charge density on the surface of particles in aqueous phase. To overcome this weakness, recently, cationic starch consisting of starch and cationic groups was developed for flocculation (Shi et al., 2016). The cationic groups could reduce the repulsive force between suspended particles and promote the flocculation process (Barakat, 2011). In addition, the increase of pH value in aqueous phase would not negatively impact the flocculating capacity of cationic starch. Therefore, cationic starch is supposed to be a promising flocculating agent for the pretreatment of AD-SM.

This study conducted turbidity reduction and ammonia stripping in the pretreatment of AD-SM. After pretreatment, the AD-SM with low dilution ratio could be used for algae cultivation and had much better performance than AD-SM with high dilution ratio in terms of biomass yield and nutrients removal. Compared with the high-dilution strategy, this pretreatment strategy could reduce the treatment period of AD-SM, increase the biomass yield, and reduce the freshwater consumption. According to the results of manure treatment and economic analysis, it is a feasible way to apply the pretreatment strategy consisting of turbidity reduction and ammonia stripping in the treatment AD-SM.

7.2. Materials and methods

7.2.1. Swine manure and algal strain

Anaerobically digested swine manure (AD-SM) was stored in refrigerator at 4 ºC. In the lab scale experiment, AD-SM was sterilized at 121 ºC for 30 min before algae inoculation. The 250 mL Erlenmeyer flasks with 100 mL AD-SM were used for algae cultivation. These flasks were shaken (150 rpm) under fluorescent lights (120±10 μmol photons m^{-2} s^{-1}) at room temperature (25±1 ºC).

The algal strain used for AD-SM treatment was *Chlorella vulgaris*. Before inoculation into AD-SM, the algae were preserved on solid artificial medium with 15% agar .

7.2.2. Parameters measurement

7.2.2.1. Nutrient profile analysis and turbidity measurement

AD-SM was centrifuged at 8000 rpm for 5 min to remove suspended solids and supernatant was collected for nutrients analysis. Ammonia nitrogen (NH_3-N), total nitrogen (TN), chemical oxygen demand (COD), and total phosphorus (TP) of AD-SM or artificial medium were measured by using analysis kits purchased from Hach Co. Ltd (USA). The measurement was performed by a spectrophotometer according to published method. Concentrations of nutrients were expressed as mg/L. Nutrients removal efficiencies were calculated according to Eq. 1.

$$R = \frac{N_0 - N_t}{t} \times 100\%$$

Eq. 1

where R is the nutrients removal efficiencies (%); N_0 and N_t are the concentrations of certain nutrients on Day 0 and Day t; t is the cultivation period (day) of algae in AD-SM.

Concentrations of short-chain fatty acids, including acetic acid, propionic acid, and butyric acid, in AD-SM were measured by using gas chromatography equipped with a flame ionization detector (GC-FID) according to the method described by Hu et al. (2012). Concentrations of short-chain fatty acids, expressed as mg/L, were calculated based on the peak areas and the calibration curves (Kong et al., 2014).

Turbidity meter was used to measure the turbidity, which was expressed as Nephelometric Turbidity Unit (NTU), of AD-SM. Suspended solids and pigment mainly contributed to the turbidity in wastewater (Wang et al., 2010).

7.2.2.2. Algae growth and biomass yield

In this work, total volatile suspended solid (TVSS), reflecting the dry weight of algae biomass, was measured according to published method . Average growth rates of algae were calculated according to Eq. 2.

$$G = \frac{W_t - W_0}{t}$$

Eq. 2

where G is the average growth rate of algae; W_t and W_0 are the dry weights of algae biomass on Day t and Day 0; t is the cultivation period (day) of algae in AD-SM.

Survival efficiency (%), which is a parameter to reflect the percentage of living algal cells in total cells, was measured with a microscope purchased from Nexcelom (USA) (Castle et al., 2011).

7.2.2.3. Composition of algae biomass

Harvested algae biomass was dehydrated in a vacuum dryer before protein content measurement and crude oil extraction (Liu et al., 2016). Protein content in algae biomass was calculated according to the total nitrogen content, which was measured by micro elemental analyzer (Morales-Sánchez et al., 2013). The nitrogen-to-protein factor (NTP) of 6.25 was used for the calculation of protein content. Detailed measurement and calculation procedures were described by Lu et al. (2015). To measure the oil content in algae biomass, ultrasound assisted oil extraction was performed according to previous publication and oil content of algae biomass was calculated accordingly.

7.2.3. Design of experiment

This work, aiming at cultivating algae in AD-SM with low dilution ratio and reducing the consumption of freshwater, consisted of four steps. First, the basic characteristics of AD-SM were measured to evaluate its feasibility for algae cultivation. Second, effects of dilution on algae growth and nutrients removal in AD-SM were assessed. Barriers to algae growth in AD-SM with low dilution ratio were identified. Third, pretreatments, including cationic starch flocculation and ammonia stripping, of AD-SM were conducted to mitigate those identified barriers. The parameters of flocculation and stripping were optimized accordingly. Fourth, three types of manure, including raw AD-SM with low dilution, pretreated AD-SM with low dilution, and AD-SM with high dilution were compared according to the nutrients removal and biomass yield in pilot scale system.

Economic analysis was conducted to assess the advantages of the integrated pretreatment developed by this work.

All the experiments and tests in this study were performed in triplicate. The results were expressed as mean ± deviation.

7.2.4. Effects of dilution on algae growth and wastewater treatment

Effects of dilution, 4, 8, 12, 16, and 20-folds, on algae growth and nutrients removal in AD-SM were assessed. The optimum dilution ratio of AD-SM for algae cultivation was determined accordingly. According to the biomass yield and nutrients removal, barriers to algae growth in AD-SM with different dilution ratios were identified.

7.2.5. Cationic starch flocculation and ammonia stripping

7.2.5.1. Cationic starch flocculation

To synthesize cationic starch, 5 g corn starch was reacted with 3 g glycidyltrimethylammonium chloride (GTAC) at 60 ºC in water bath for 5 hours with 1.5 mL NaOH solution (1 mol/L) as catalyst (Şen et al., 2017). After that, excessive ethanol was added to promote the polymer sedimentation and then the precipitated polymer was dehydrated in an oven at 60 ºC for 10 hours (Yanling et al., 2016). Dry polymer was stored in dark at 4 ºC before being used as flocculating agent for AD-SM pretreatment.

The flocculation was performed by adding certain amounts (0, 0.1, 0.2, 0.3, and 0.4 g) of cationic starch in 1 L AD-SM with 4-fold dilution and mixing for 2 min. After that, AD-SM was subjected to settlement. To save energy and reduce cost, in this work, settlement

was driven by gravity. Turbidities of supernatants at different settlement time were measured. After settlement, the supernatant was collected for subsequent experiment.

7.2.5.2. Ammonia stripping

Algae were cultivated in artificial simulated medium with different ammonia concentrations (0, 100, 200, 300, 400, and 500 mg/L) to evaluate the threshold of ammonia toxicity. The cultivation periods were 5 days. Expected ammonia concentration for algae cultivation was identified according to average growth rate and survival efficiency of algae.

Air bubbling was used to strip ammonia from AD-SM with 4-fold dilution in a 4 L bottle at room temperature (25±1 ^{o}C). As shown in Figure 7.1, ionized ammonium and dissolved ammonia, two major forms of ammonia, reached dynamic equilibrium in waste effluents. Such a dynamic equilibrium is impacted by the temperature, pH value, concentrations of ions, and many other factors (El-Bourawi et al., 2007). Under specific condition, the ratio of ionized ammonium to dissolved ammonia is a constant. The mechanism of ammonia stripping assisted by air bubbling is that air flow takes out a portion of dissolved ammonia and disturbs the dynamic equilibrium between ionized ammonium and dissolved ammonia (Ferraz et al., 2013). As a result, to reach a new dynamic equilibrium, a portion of ionized ammonium is converted to dissolved ammonia (Liu et al., 2015). Therefore, air bubbling could effectively reduce the concentration of total ammonia in aqueous phase by removing dissolved ammonia and creating a new dynamic equilibrium.

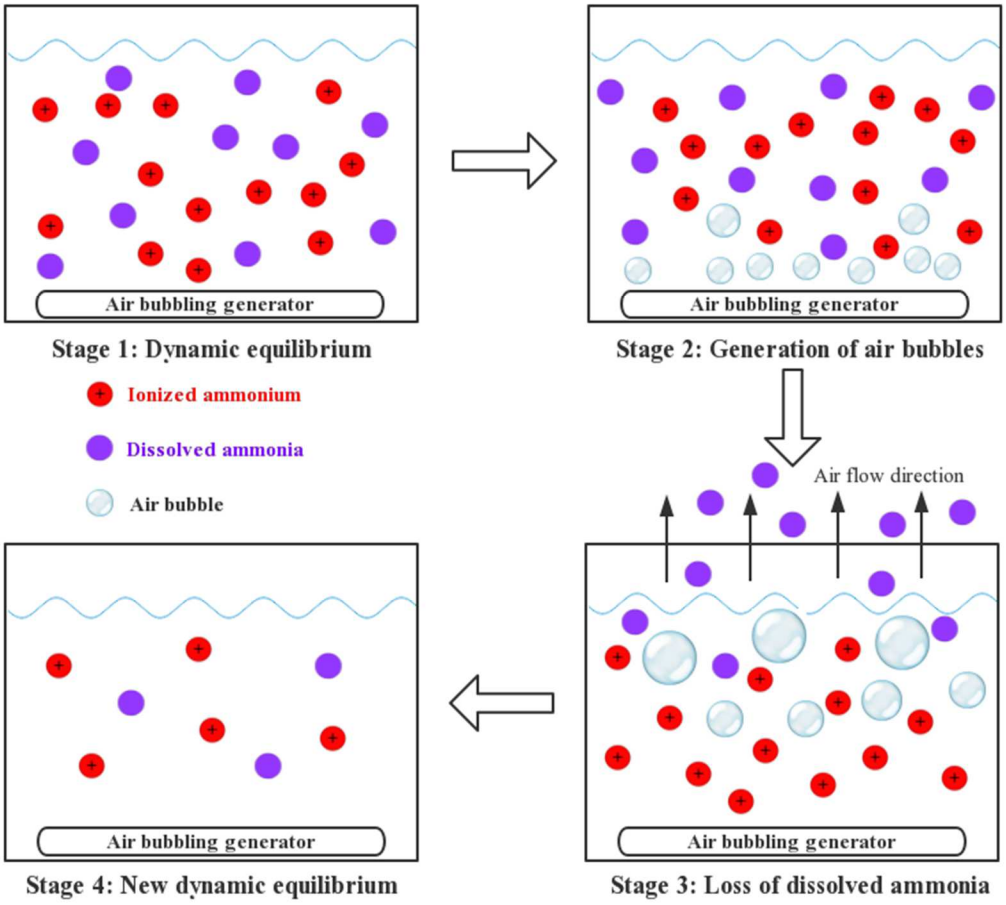


Figure 7.1. Mechanisms of ammonia stripping process assisted by air bubbling

The optimum stripping time was identified according to the achievement of expected ammonia concentration in AD-SM. Considering the low cost of air bubbling treatment, this method should be economically feasible in the practice.

7.2.6. Treatment of AD-SM in pilot scale system and economic analysis

Three types of AD-SM, including raw AD-SM with 4-fold dilution, pretreated AD-SM with 4-fold dilution, and AD-SM with 16-fold dilution, were used for algae cultivation in a pilot scale system (about 1500 L), consisting of a retention tank (about 300 L) and multi-layers of photo-bioreactors. This bioreactor was located in a greenhouse of which

the light source was sunlight. About 700 L original AD-SM was used to cultivate algae in each way. Considering the unfavorable conditions, such as darkness at night and temperature fluctuation, batch cultivation periods of algae in pilot scale system were extended to 8 days. Biomass yield of algae and nutrients removal in AD-SM were measured daily.

Facility investment, time, energy input, and material input were quantitatively recorded for economic analysis (Xin et al., 2016). The facilities mainly included greenhouse, bioreactor, flocculation tank, and ammonia stripping device. Electricity consumption, which is the major energy input, was caused by the operation of bioreactor and some other devices. Material input included freshwater and cationic starch. According to the economic analysis, the unit costs and energy inputs of algae cultivation in three types of AD-SM were calculated and compared.

7.3. Results

7.3.1. Characteristics of AD-SM

As shown in Table 7.1, compared with artificial medium, AD-SM from farm contained much more essential nutrients for algae growth. Concentrations of NH_3-N, TN, TP and COD in AD-SM were 1795.04%, 568.05%, 67.42%, and 155.17% higher than those in artificial medium, respectively. In addition, in both AD-SM and artificial medium, the dominant organic carbon was acetate, which is a good carbon source for algae growth (He et al., 2017). The neutral value of pH in swine manure is another factor that is favorable to algae cultivation.

Table 7.1. Characteristics of AD-SM and artificial medium

Parameter	AD-SM	Artificial medium
TVSS (g/L)	1.645±0.235	0
pH	6.82±0.19	7.05±0.36
NH_3-N (mg/L)	1874.9±6.7	128.9±3.1
TN (mg/L)	2534.5±15.6	379.4±15.5
TP (mg/L)	53.7±2.6	32.1±2.9
COD (mg/L)	9876.2±72.8	3871.4±98.6
Acetic acid (mg/L)	1722.75±68.23	1089.52±59.64
Propionic acid (mg/L)	919.47±38.71	0
Butyric acid (mg/L)	214.85±12.88	0

Although some nutrients are essential to algal metabolisms, excessive concentrations may limit algae growth or even cause the failure of algae cultivation. For example, concentration of NH_3-N in AD-SM reached 1874.95 mg/L, which was much higher than the threshold of ammonia toxicity to most algal species. Lu et al. (2018) reported that in artificial wastewater, algae growth was prohibited when the concentration of NH_3-N exceeded 392 mg/L. Besides ammonia toxicity, high content of suspended solids, which could seriously reduce the light transmission and further limit the photosynthesis rate of algal cells, in AD-SM might be another unfavorable factor (Hjorth et al., 2008).

According to the discussion above, it was hypothesized that although AD-SM contained essential nutrients and had neutral pH value, it might not be directly used for algae cultivation due to some limiting factors.

7.3.2. Algae cultivation in diluted AD-SM

Figure 7.2(a) indicated that no algae growth was observed in original AD-SM and the survival efficiencies of algal cells decreased gradually with the extension of cultivation period. This result confirmed the hypothesis that original AD-SM could not be directly used for algae cultivation. To mitigate the limiting factors, in previous studies, AD-SM was diluted appropriately before algae inoculation (Hu et al., 2012). Hu et al. (2012) reported that algae had the highest biomass yield (about 0.6 g/L) in AD-SM with 20-fold dilution. In some studies, the dilution ratios of AD-SM were even higher than 25-fold (De la Noüe & Basseres, 1989; Zhou et al., 2012a). As shown in Figure 7.2(b), biomass yields of algae grown in AD-SM with 4-fold, 8-fold, 12-fold, 16-fold, and 20-fold dilution reached 0.389, 0.521, 0.546, 0.578, and 0.502 g/L, respectively. In terms of biomass yield, 16-fold was the optimum dilution ratio. Not only the biomass yield, but also survival efficiency of algal cells reached peak value (86.4%) when the dilution ratio of AD-SM was 16-fold (Figure 7.2(c)). Therefore, dilution in certain range could alleviate some limiting factors in AD-SM and promote algae growth (Hu et al., 2012).

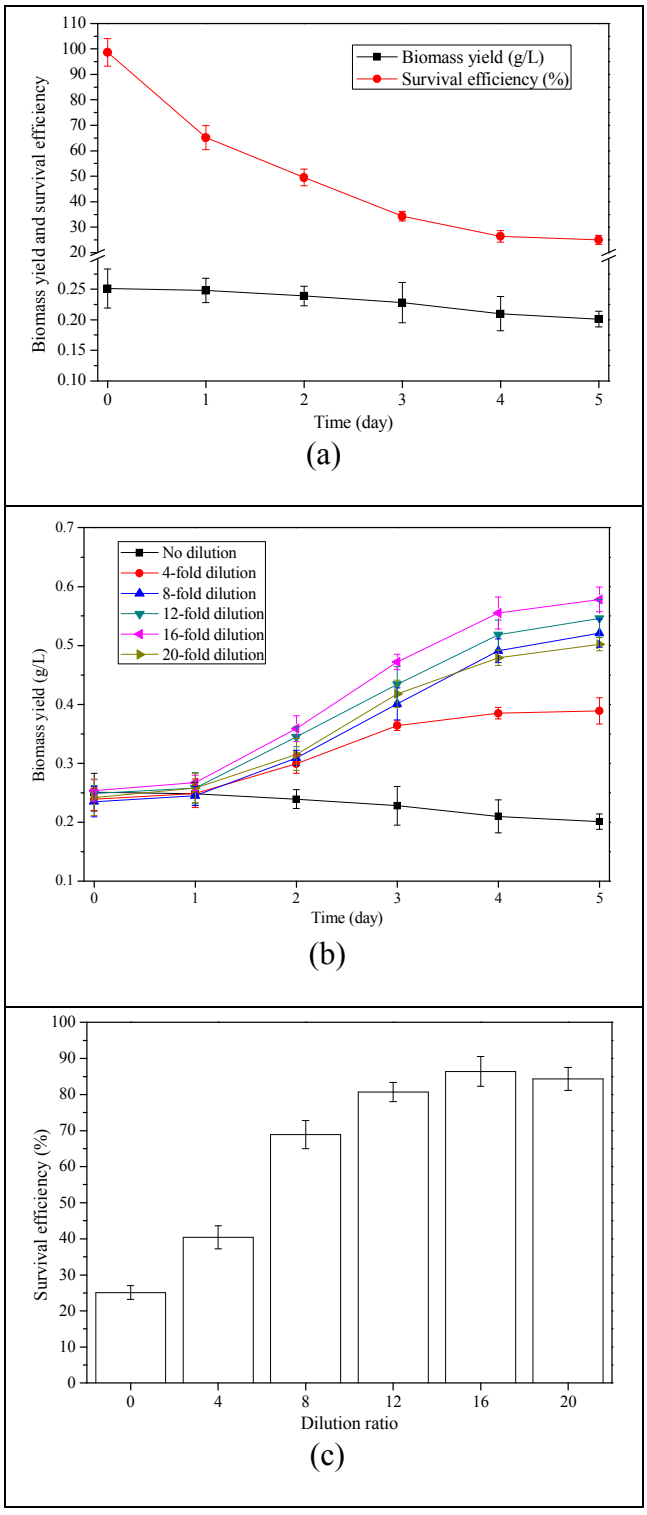


Figure 7.2. Algae growth and nutrients removal in AD-SM

With the increase of dilution ratios, initial concentrations of nutrients decreased. As a result, some nutrients in diluted AD-SM were not sufficient to support algae growth. For

example, when the dilution ratios of AD-SM exceeded 4-fold and 8-fold, removal efficiencies of COD and NH_3-N approached 100%, respectively. Therefore, when the dilution ratio was higher than 4-fold, nutrient deficiency would become a problem limiting algae growth and biomass production. However, in the AD-SM with 4-fold dilution ratio, the biomass yield of algae was not high (0.389 g/L) although there were sufficient nutrients. Biomass yield of algae in the AD-SM with 4-fold dilution ratio was 34.95% lower than that in the AD-SM with 16-fold dilution ratio. Hence, besides nutrients availability, other factors may impact algae growth in AD-SM.

In AD-SM with 4-fold dilution, although the nutrients were sufficient, due to the low assimilation rate of inorganic carbon by photosynthesis, biomass yield was lower than that in highly diluted AD-SM (Gupta et al., 2016). In addition, in AD-SM with 4-fold dilution, ammonia toxicity is another limiting factor to algae growth (Markou et al., 2016). In some cases, excessive ammonia in wastewater or culture medium could also negatively impact the oil quality of algal biomass by causing oxidative stress (Nimptsch & Pflugmacher, 2007). Therefore, to cultivate algae in AD-SM with 4-fold dilution, turbidity and ammonia toxicity should be considered. Based on the discovered problems, two strategies were proposed to pretreat the AD-SM. The first strategy, which has been reported by many studies, is pretreating AD-SM by high dilution (De la Noüe & Basseres, 1989; Hu et al., 2013). The second strategy is removing turbidity and reducing ammonia concentration in AD-SM with low dilution ratio.

7.3.3. Turbidity reduction and ammonia stripping

As shown in Figure 7.3(a), turbidity of supernatant was reduced with the increase of cationic starch content. After 50 min settlement, turbidity reduction efficiencies in AD-SM added with 0, 0.1, 0.2, 0.3, and 0.4 g/L cationic starch were 4.38%, 44.35%, 79.17%, 83.42%, and 88.59%, respectively. When the cationic starch content exceeded 0.2 g/L, it was not an effective way to remove turbidity in AD-SM by further increasing cationic starch content. For example, residual turbidity was only reduced by 104.9 NTU when cationic starch content increased from 0.2 to 0.4 g/L (Figure 7.3(a)). In addition, the residual turbidity (234.3 NTU) of AD-SM pretreated by 0.2 g/L cationic starch was low enough to support the algae growth (Van Den Hende et al., 2014). Hence, the content of cationic starch for turbidity reduction was set as 0.2 g/L.

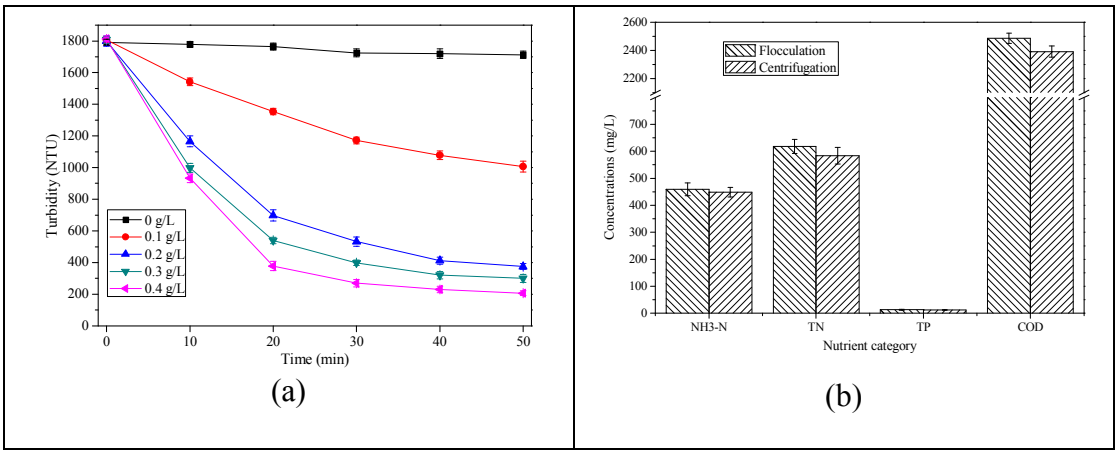


Figure 7.3. Turbidity reduction by cationic starch and changes of nutrients profile

Figure 7.3(a) showed that in AD-SM added with 0.2 g/L cationic starch, turbidity of supernatant decreased by 77.10% in 40 min while only decreased by 2.06% between 40 min and 50 min. This result is also confirmed by Figure 7.4. Therefore, the settlement time of turbidity reduction was set as 40 min. Figure 7.3(b) showed that the AD-SM after flocculation and centrifugation had the similar nutrient profiles, suggesting that turbidity

reduction by cationic starch mainly caused the settlement of suspended solids while did not remove the soluble nutrients.

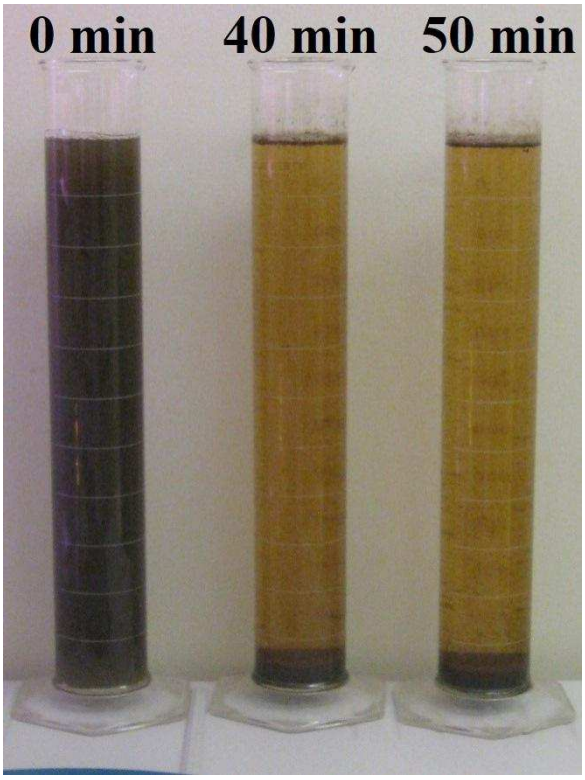


Figure 7.4. Picture of AD-SM added with 0.2 g/L cationic starch at different settlement time

The expected ammonia concentration was identified according to Figure 7.5(a), which showed that algae had the highest growth rate (0.204 g/L/day) when the concentration of ammonia was 300 mg/L. This result was in accordance with the optimum concentration of ammonia reported by previous study. In addition, survival efficiency of algal cells dropped when the concentration of ammonia exceeded 300 mg/L. Therefore, in this work, the expected ammonia concentration for algae growth was 300 mg/L. The purpose of ammonia stripping was to reduce the concentration of ammonia in AD-SM with 4-fold dilution to 300 mg/L.

Figure 7.5(b) indicated that both air flow rate and stripping time impacted the removal of ammonia in AD-SM. Although ammonia volatilization was accelerated with the increase of air flow rate, ammonia removal efficiency and air flow rate were not in a unary linear regression relationship. Removal efficiencies of ammonia reached 7.91%, 22.21%, 34.91%, 42.05%, 47.62%, and 51.32%, respectively, when the air flow rates were 1, 2, 4, 6, 8, and 10 L/min. This result suggested that when the air flow rate exceeded 6 L/min, the increase of ammonia removal efficiency slowed down. To reduce the energy consumption, hence, the air flow rate for ammonia stripping was set as 6 L/min. It was also observed that the removal of ammonia mainly occurred in the first 4 hours (Figure 7.5(b)). For example, when the air flow rate was 6 L/min, 29.27% of ammonia was removed from 0-4 h while only 12.78% of ammonia was removed from 4-8 h. The main reason is that in aqueous phase with higher concentration of total ammonia in certain range, more ammonia was in the form of dissolved ammonia. Accordingly, the air bubbling treatment stripped ammonia in a more efficient way during first two hours. However, after 4 h, the concentration of dissolved ammonia was much lower. Accordingly, the air bubbling treatment took out much less ammonia from AD-SM and the removal efficiency was reduced. Similar phenomenon was also reported in previous studies that stripped ammonia from landfill leachates and anaerobic fermentation wastewater by air bubbling (Smith & Arab, 1988). As shown in Figure 7.5(b), to reduce the concentration of ammonia in AD-SM with 4-fold dilution to 300 mg/L, stripping time should be controlled at 5 h.

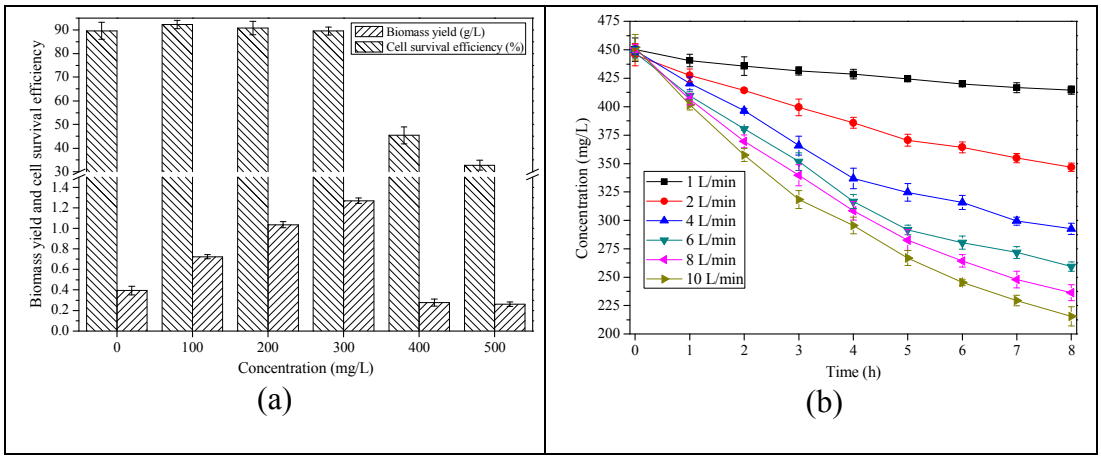


Figure 7.5. Ammonia stripping to mitigate ammonia toxicity in AD-SM

According to the discussion above, pretreatment conditions for AD-SM were: 0.2 g/L cationic starch and 40 min settlement for turbidity reduction and 6 L/min air flow rate for ammonia stripping (5 h).

7.3.4. Algae cultivation in pretreated AD-SM with 4-fold dilution

7.3.4.1. Algae growth and nutrients removal

Figure 6(a) showed that compared with the biomass yield in raw AD-SM, that in pretreated AD-SM increased by 266.58%. With the mitigation of barriers, biomass yield (1.626 g/L) in pretreated AD-SM with 4-fold dilution was even much higher than that (1.065 g/L) in artificial medium. Therefore, pretreatment by turbidity reduction and ammonia stripping effectively promoted the algae growth, making AD-SM a better effluent for biomass production than artificial medium.

As shown in Figure 6(b) and Figure 6(c), removal efficiencies of NH_3-N, TN, TP, and COD in pretreated AD-SM with 4-fold dilution reached 91.57%, 80.24%, 78.57%, and

89.74%, respectively. Compared with those in raw AD-SM with 4-fold dilution, removal efficiencies of NH_3-N, TN, TP, and COD increased by 30.53%, 22.46%, 13.42%, and 21.71%, respectively. One of the main reasons for the higher removal efficiencies is that algae with better growth in pretreated AD-SM assimilated more nutrients. At the end of cultivation, concentrations of residual NH_3-N, TN, TP, and COD were 23.7, 91.2, 2.4, and 217.9 mg/L, meeting the requirement of wastewater discharge standard . This result demonstrated that the pretreatment of AD-SM not only generated economic benefits by producing more biomass, but also generated environmental benefits by promoting nutrients recycling.

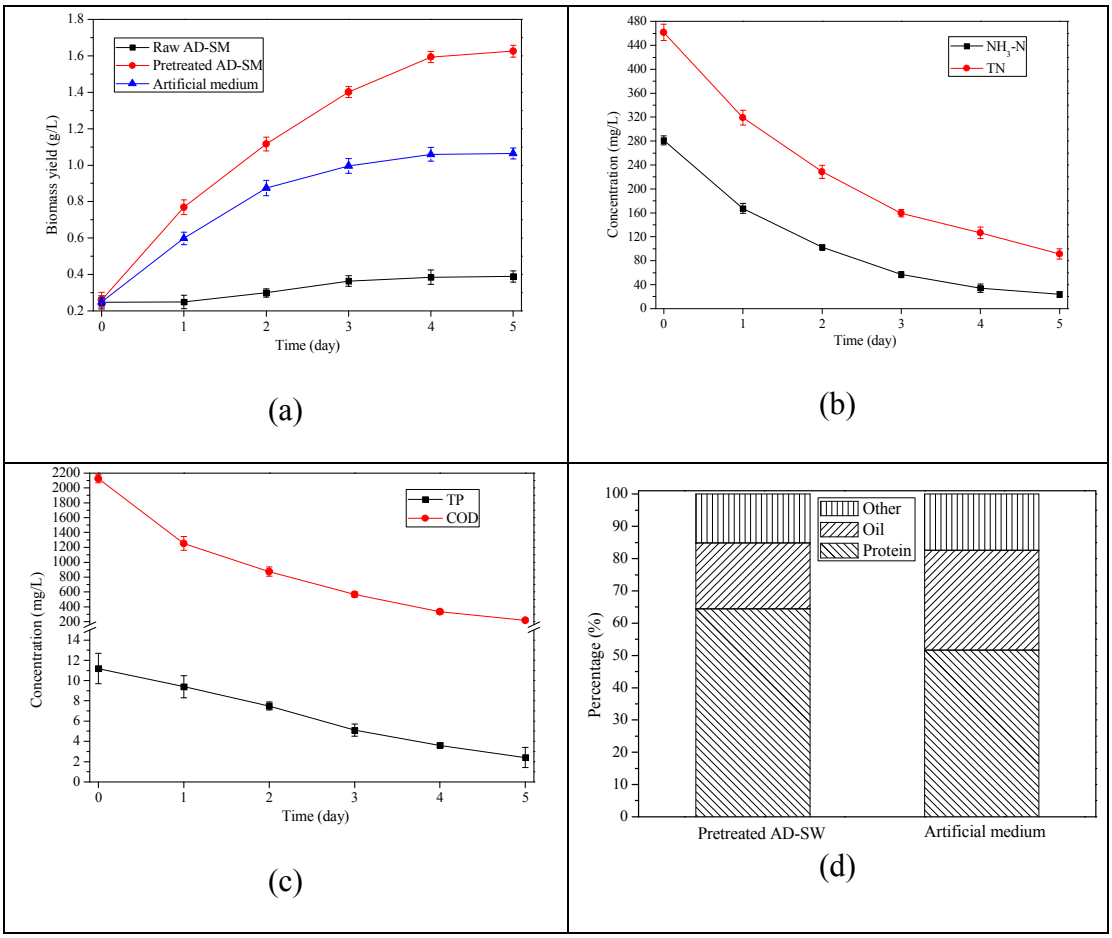


Figure 7.6. Algae growth and nutrients removal in AD-SM with 4-fold dilution and artificial medium

7.3.4.2. Composition of algal biomass

Algae biomass harvested from the pretreated AD-SM with 4-fold dilution contained 64.4% protein and 20.4% oil (Figure 6(d)). Due to the high protein content, the algae biomass could be exploited as animal feed or bio-fertilizer. Compared with the biomass harvested from artificial medium, the biomass from pretreated AD-SM contained more protein but less oil. The main reason is that ammonia concentration in pretreated AD-SM was about 187.04% higher than that in artificial medium. Sufficient ammonia was favorable to the protein synthesis in algal cells. In addition, in artificial medium with lower turbidity, algal cells had better performance in photosynthesis, which is one of the main pathways for oil synthesis (Koller et al., 2012). Therefore, algae harvested from pretreated AD-SM and artificial medium had different nutrient compositions.

7.3.5. Comparison of pretreatment strategies and economic analysis

7.3.5.1. Algae cultivation in pilot scale system

In pilot scale system, biomass of algae grown in raw AD-SM with 4-fold dilution, pretreated AD-SM with 4-fold dilution, and AD-SM with 16-fold dilution reached 0.347, 1.626, and 0.532, respectively (Table 7.2). In terms of biomass yield, pretreated AD-SM with 4-fold dilution was the best one for algae cultivation. Interestingly, it was observed that in each type of AD-SM, biomass yield in pilot scale system was lower than that in lab scale experiment. For example, in pretreated AD-SM with 4-fold dilution, biomass yield of algae grown in lab was 4.07% higher than that of algae grown in pilot scale

system. Similar phenomenon was also reported by previous study (Pérez-López et al., 2014). The unfavorable condition in greenhouse was the main reason for such a phenomenon.

Table 7.2. Algae growth and nutrients removal in pilot scale system with three types of AD-SM

Items	Raw AD-SM with 4-fold dilution		Pretreated AD-SM with 4-fold dilution		AD-SM with 16-fold dilution	
	Residual (mg/L)	Removal	Residual (mg/L)	Removal	Residual (mg/L)	Removal
NH_3-N	243.7	46.27%	27.8	90.12%	0	100%
TN	332.8	47.66%	141.6	69.32%	23.6	84.85%
TP	5.9	55.30%	1.2	89.29%	0	100%
COD	1134.5	54.33%	294.6	86.13%	23.5	96.23%
Biomass yield (g/L)	0.347		1.597		0.532	

7.3.5.2. Economic analysis

Economic analysis indicated that pretreated AD-SM with 4-fold dilution was the best one for algae cultivation according to the unit energy input, cost, and freshwater consumption (Table 7.3). Since ammonia stripping system and flocculation tank were needed for the pretreatment, the facility cost ($7549) of using pretreated AD-SM with 4-fold dilution was slightly higher than the costs of using other two types of AD-SM. In the research of Xin et al. (2016) that focused on the techno-economic analysis of wastewater-based

biomass production, the costs of facilities, including greenhouse and bioreactor, were much higher than those reported by this work. For example, Xin et al. (2016) claimed that the cost of greenhouse was higher than $430000 while the cost of greenhouse in this study was only about $3400. Different parameters of facilities used by this work and previous studies also caused the difference of cost (Medeiros et al., 2015).

Table 7.3. Economic analysis for algae cultivation in three types of AD-SM

	Items	Raw AD-SM with 4-fold dilution	Pretreated AD-SM with 4-fold dilution	AD-SM with 16-fold dilution
Facility	Greenhouse	$3476	$3476	$3476
	Bioreactor (1500 L)	$2975	$2975	$2975
	Ammonia stripping system	/	$650	/
	Flocculation tank (500 L)	/	$448	/
	Summary	$6451	$7549	$6451
Time	Volume	2800 L	2800 L	11200 L
	Treatment batch	2	2	8
	Time	16 days	17 days	64 days
Energy input	Operation of greenhouse	25.6 kW·h	25.6 kW·h	102.4 kW·h
	Operation of bioreactor	51.2 kW·h	51.2 kW·h	204.8 kW·h
	Air bubbling device	/	6.0 kW·h	/
	Mixing device for flocculation	/	0.8 kW·h	/
	Summary	76.8 kW·h	83.6 kW·h	307.2 kW·h
Material input	Cationic starch	/	0.60 kg	/
	Freshwater	2100 L	2100 L	10500 L
Other fees	Labor salary	$350	$370	$1400
	Post-treatment	$400	/	/
	Land utilization fee	$105	$112	$420
Unit cost/ input	Unit energy input	0.105 kW·h/g dry biomass	0.030 kW·h/g dry biomass	0.052 kW·h/g dry biomass
	Unit freshwater consumption	2.88 L/g dry biomass	0.75 L/g dry biomass	1.76 L/g dry biomass

Since 700 L original AD-SM yielded 11200 L AD-SM at 16-fold dilution, it was necessary to treat the AD-SM in 8 batches. However, it only took 2 batches to treat the AD-SM with 4-fold dilution. Accordingly, the time (64 days) of treating AD-SM with 16-fold dilution was much longer than that (16 or 17 days) of treating AD-SM with 4-fold dilution (Table 7.3). In the practice, long treatment period would increase the operation cost and seriously reduce the unit treatment capacity of the wastewater treatment plant. Therefore, saving time is one of the great advantages of low dilution strategy for algae-based AD-SM treatment.

The energy consumption was mainly caused by the operation of greenhouse and bioreactor. In this work, the average electricity consumption each day was about 4.8 kW·h. Total electricity consumption of the pretreatment by turbidity reduction and ammonia stripping was 6.8 kW·h, so the pretreatment only slightly increased the electricity consumption. Due to the long cultivation time, electricity input of AD-SM with 16-fold dilution was 267.46% higher than that of pretreated AD-SM with 4-fold dilution. Besides the energy input, low dilution effectively reduced the freshwater consumption. As shown in Table 7.3, the freshwater consumption of AD-SM with 4-fold dilution was only 20% of the freshwater consumption of AD-SM with 16-fold dilution. Accordingly, the cost caused by freshwater consumption was reduced by the low dilution strategy. Since freshwater is a valuable resource in the nature, low freshwater consumption will also reduce the footprint of algae cultivation and generate environmental benefits (Yang et al., 2011b).

Based on the data in Table 7.2 and Table 7.3, it was summarized that algae cultivated in pretreated AD-SM with 4-fold dilution had the lowest unit energy cost ($0.005/g dry

biomass) and unit freshwater consumption (0.75 L/g dry biomass). Although the total energy input of using raw AD-SM was lower than that of using pretreated AD-SM, low biomass yield in raw AD-SM increased the unit energy input and unit energy cost. The unit energy input of using pretreated AD-SM with 4-fold dilution was 42.31% lower compared with that of using AD-SM with 16-fold dilution. In addition, the unit freshwater consumption of using pretreated AD-SM with 4-fold dilution was 57.39% lower than that of using AD-SM with 16-fold dilution. Therefore, it has great advantages to use pretreated AD-SM with low dilution for algae cultivation.

7.4. Conclusions

It is concluded that (1) High turbidity and ammonia toxicity are two barriers to algae growth in AD-SM with low dilution; (2) High dilution is an effective way to mitigate these barriers, but it could not be widely applied due to the high energy input, long treatment time, and high freshwater consumption; (3) Cationic starch effectively flocculated suspended solids in AD-SM and reduced turbidity; (4) 6 L/min air flow rate and 5 h stripping time were regarded as good conditions for ammonia stripping; (5) Biomass yield of algae in pretreated AD-SM with 4 –fold dilution reached 1.597 g/L and the AD-SM was dischargeable after algae cultivation; (6) According to the economic analysis, it has great advantages to use pretreated AD-SM with low dilution for algae cultivation.

Chapter 8. Summary and future work

8.1. Summary of the dissertation

Wastewater-based algae cultivation has been considered as a promising way to produce biomass and treat wastewater. However, some technical problems limited the wide application of wastewater in algae cultivation. This dissertation research mainly focused on three problems, including unbalanced nutrients profile, ammonia toxicity, bacterial contamination, associated with wastewater-based algae cultivation. The main objective of this dissertation research was to mitigate some technical problems and promote the use of algae in wastewater treatment.

The Chapter 3 and Chapter 4 identified one of the serious bottlenecks to algae growth in dairy processing wastewater and meat processing wastewater. The bottleneck to algae growth was mitigated by mixing wastewater from different sources. This strategy avoided the use of any artificial chemical, ammonium chloride, to balance the nutrients profile of wastewater. Accordingly, the production cost of algae biomass could be reduced. In addition, acid hydrolysis was conducted to convert solids in wastewater to soluble nutrients. After hydrolysis, nutrients released from solids could be used for algae cultivation. The strategies based on nutrients balancing were favorable to the full exploitation of nutrients in wastewater.

Chapter 5, which explored the ammonia toxicity in wastewater, proposed a strategy to alleviate ammonia toxicity to algae growth. Comparison between bicarbonate, citric acid, and glucose suggested that glucose had the best performance in the alleviation of ammonia toxicity. The potential reason for this phenomenon is that the carbon source should provide enough energy for ammonia assimilation, which is an energy-consuming

process. In the practice, some wastewater with high concentration of glucose could be used to cultivate algae and mitigate potential ammonia toxicity.

In Chapter 6, the cooperation between algae and bacteria was observed in municipal wastewater. Existence of aerobic bacteria did not cause the failure of algae cultivation, but increased biomass yield of algae. The co-existence of algae and bacteria also promoted the removal of nutrients. The results of high throughput sequence analysis indicated that aerobic bacteria became dominant at the end of cultivation period. An aerobic bacterium, *Acinetobacter* sp. was isolated and co-cultivated with algae together. According to the metabolisms of aerobic bacteria and algae, in this work, the cooperation between algae and wastewater-borne bacteria was attributed in part to the exchange of carbon dioxide and oxygen between bacteria and algae.

Chapter 7 developed a combined strategy of cationic starch-assisted turbidity reduction and air bubbling-driven ammonia stripping for the pretreatment of anaerobically digested swine manure. The results showed that after pretreatment, the anaerobically digested swine manure became a good effluent for algae cultivation. According to the economic analysis in pilot-scale system, compared with some traditional pretreatment strategies, this strategy had many advantages, such as lower cost, less energy input and simpler procedure.

8.2. Future work

This dissertation research devoted a lot of effects in the exploitation of wastewater for algae cultivation. Some strategies were proposed to solve the problems of ammonia toxicity and unbalanced nutrients profile in wastewater and a cooperation model between

algae and wastewater-borne bacteria was discovered. To further promote the commercialization of wastewater-based algae cultivation, some work are needed in the future.

First, more research should be conducted to explain the mechanisms of ammonia toxicity from the perspective of cell metabolisms. Based on the response of algal metabolisms to ammonia toxicity, more endogenous approaches could be developed to promote assimilation of ammonia by algal cells in wastewater. For example, genetic modification could be conducted in algal cells to enhance the performance of GS-GOGAT pathway. In this work, the strategy based on carbon supply has high requirement on the cultivation conditions and has high energy consumption. To reduce the production cost, in the practice, endogenous approaches based on genetic modification could be developed to support the ammonia assimilation.

Second, it is necessary to confirm the strategies developed by this study in the large scale system. Since there is difference between lab scale experiment and large scale wastewater treatment, strategies developed in lab may not be used directly in the large scale system for wastewater treatment. In the future, to use the strategies developed by this dissertation research in large scale system, more effects should be devoted.

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